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EFFECTS OF SOME *ISO*THIOUREA AND GUANIDINE SALTS ON VARIOUS PREPARATIONS OF SMOOTH AND STRIPED MUSCLE

BY

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(Received July 4, 1949)

In seeking pressor agents of new types, Smirk (1941) was led to test the fairly well known organic chemical methyl *iso*thiourea sulphate. He found it capable of producing large persistent rises of blood pressure in anaesthetized animals. According to McGeorge, Sherif, and Smirk (1942), the rises of blood pressure are brought about mainly if not entirely through a direct action of methyl *iso*thiourea on blood vessels; and this action is not sympathomimetic. They state that the pressor action was hardly affected by the anti-adrenaline compounds, ergotoxine and F933. Methyl *iso*-thiourea, unlike adrenaline, tended to increase the tone of isolated muscle strips from rabbit intestine, bladder, and uterus.

Certain of its chemical relatives have been found to behave similarly: the pharmacological properties of methyl *iso*thiourea are reproduced with most fidelity by amidine derivatives like ethyl *iso*urea and guanidine salts which ionize freely to yield cations of small size (Fastier and Smirk, 1943 and 1947; Fastier, 1948). It would appear that pressor analogues of methyl *iso*thiourea need have nothing more in common with it *structurally* than the amidine group

$$\left(-C\langle^{NH-}_{NH-}\right)^{+}$$

Now histamine contains this group. Moreover, it is known that histamine too causes the contraction of most kinds of smooth muscle irrespective of the innervation. The possibility that amidine derivatives like methyl *iso*thiourea and guanidine produce some of their more characteristic pharmacological effects through simulating actions of histamine has therefore appeared worthy of investigation.

Other experiments reported below were suggested by a much older idea—one which goes back to Führer (1908), who compared the action of guanidine on striped muscle to that of a univalent alkali metal cation. He pointed out that the effects of both are antagonized by such divalent cations as calcium. Similar observations on the interaction of calcium and guanidine have been published by a number of workers (Major and Stephenson, 1924; Ochoa, 1928; Minot, 1931; Burns and Secker, 1935; Harvey, 1940). Minot, Dodd, and Riven (1938) express what is probably a widely held view when they write: "It is possible that guanidine exerts its action on muscles through changes in the effect of inorganic salts." For this reason, the effect of changes in calcium or in potassium ion concentration on the response to typical amidine derivatives has also been studied.

METHODS

Rabbits' ears were perfused at room temperature with Ringer-Locke solution kept at a constant head of pressure by means of a Mariotte bottle (Kravkov-Pissemski preparation). The venous outflow was measured by the method of Stephenson (1948). Amidine derivatives were injected as salts in neutral solution. The *iso*thioureas tested were some of those specially synthesized for this series of investigations. Samples of guanidine hydrochloride, methylguanidine sulphate, and *asym*-dimethylguanidine sulphate were obtained from commercial sources, as were also such drugs as "Priscol" and *d*-tubocurarine chloride.

Isolated strips of rabbit or guinea-pig ileum and uterine horns of non-pregnant rats were set up in an ordinary organ bath of 100 ml. capacity. The Ringer-Tyrode solution in the bath was aerated with oxygen containing 5 per cent carbon dioxide.

The bronchiole preparation employed was that of Konzett and Rössler (1940). Female guinea-pigs of 500–600 g. weight were deeply anaesthetized by injecting urethane intraperitoneally. Cannulae were inserted into a jugular vein and the trachea. The lungs were insufflated with a small Starling respiration pump

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at each stroke of which the excess air from the pump was driven into a float recorder. When the bronchioles were constricted the average level of the marker was raised (see Fig. 3).

Rectus abdominis muscle was taken from winter frogs (*Rana temporaria*). Each piece was suspended in a 5 ml. bath through which air was bubbled slowly. Isotonic contractions were recorded with a gimbal lever. The Ringer solution used contained 0.7 g. NaCl, 0.014 g. KCl, 0.012 g. $CaCl_2$, and 0.02 g. $NaHCO_3$ per 100 ml. Acetylcholine was added in known concentration (usually a 1 in 2,000,000 solution), replacing the ordinary Ringer solution of the bath for a period of 90 seconds. The muscle strip was washed two or three times after each dose of acetylcholine. The interval between doses was kept at 5 minutes.

The rat diaphragm preparation was set up as specified by Bülbring (1946). At first the bath temperature was kept at 39° C., but later a temperature of 28° C. was chosen as more suitable. Drug solutions were added directly to the bath. A "square wave" stimulator was employed; its construction permitted the amplitude, duration, and frequency of the shocks to be varied independently. The nerve was stimulated in the fluid from platinum contacts.

Experiments on cat gastrocnemius muscle were performed on animals under chloralose anaesthesia. The muscle was stimulated via the sciatic nerve with condenser shocks strong enough to give a maximal response. Contractions were recorded by attaching a stout wire from an isometric lever which wrote on a slowly moving drum to the lower end of the tendo Achillis. The central attachment of the muscle had previously been fixed firmly in position by clamping a steel rod driven through the condyles of the left femur. Drug solutions were forced into the left iliac artery through a cannula whose tip lay just below the bifurcation of the aorta.

Results

Effects on smooth muscle preparations

Perfused rabbit ear

Methyl *iso*thiourea was tested on eighteen perfused ears. A dose of 0.1 ml. of an M/5,000 solution brought about definite vasoconstriction (Fig. 1*b*) in six of eight experiments. Strong vasoconstriction was caused in all of seventeen ears by injecting 0.1 ml. of an M/1,000 solution. When 0.1 ml. of an M/100 solution was injected, its effect was intense.

Smaller vasconstrictor effects were obtained as a rule when *asym*-dimethylguanidine was given in corresponding amount. Guanidine itself was less active still.

Effect of "Priscol." — In ten experiments benzyliminazoline ("Priscol") hydrochloride was perfused as a 1 : 5,000 solution in Ringer-Locke after control injections of methyl *iso*thiourea and of adrenaline had been given. Within a few minutes the response to even a large (0.1 μg.) dose of adrenaline was "reversed." Nevertheless, vasoconstriction was still brought about when methyl *iso*thiourea was injected (Fig. 1*d*). Its action did not seem to be affected appreciably by the priscol: nor did that of guanidine or of *asym*-dimethylguanidine.

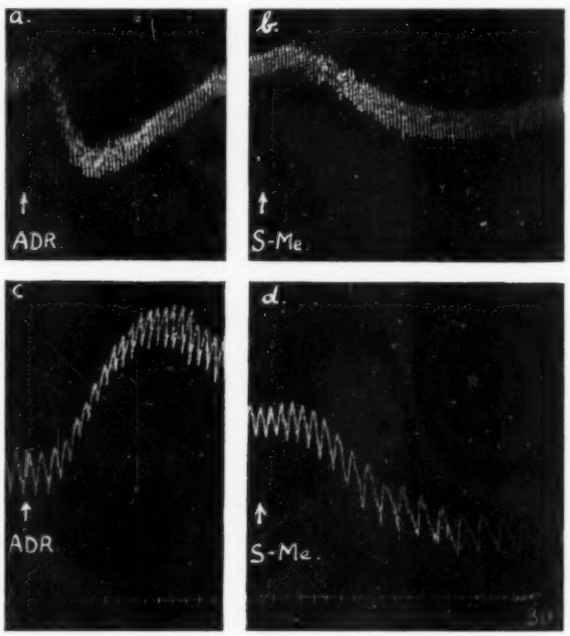

FIG. 1.—Perfused rabbit ear preparation. Upper tracings show the normal effect of (*a*) adrenaline (0.01 μg. at ADR) and (*b*) methyl *iso*thiourea (0.1 ml. of M/5,000 at S-Me). Lower tracings show the effect of (*c*) adrenaline (0.1 μg. at ADR) and (*d*) methyl *iso*thiourea (0.1 ml. of M/5,000 at S-Me) after a 1 : 5,000 solution of priscol has been perfused for about ten minutes.

Effect of "Anthisan."—Unlike priscol, the antihistamine compound N - *p*- methoxybenzyl - N - dimethylaminoethyl - α - aminopyridine ("Anthisan") maleate antagonized the vasoconstrictor response to methyl *iso*thiourea in concentrations which left the blood vessels still fairly responsive to adrenaline. In all of seven experiments it soon diminished the response to a test dose of methyl *iso*thiourea when perfused in M/1,000 solution (Fig. 2). Ultimately even so large a dose as 0.1 ml. of M/10 methyl *iso*thiourea failed to produce vasoconstriction. Slight vasodilatation was obtained occasionally with the *iso*thiourea, but there was nothing comparable to the "reversal" seen with adrenaline after priscol

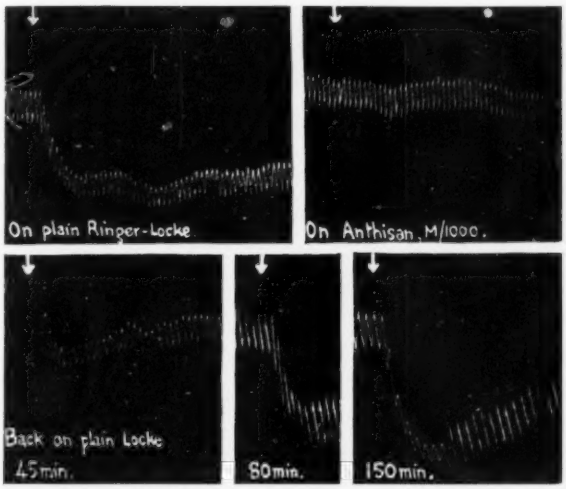

FIG. 2.—Perfused rabbit ear preparation. Methyl *iso*thiourea sulphate (0.1 ml. of M/100 given at the arrows). Its effect is practically suppressed when anthisan (M/1,000) is added to the perfusing Ringer-Locke solution. Even a very large dose of methyl *iso*thiourea (0.1 ml. of M/10 at the dot) produces hardly any vasoconstriction.

Anthisan in this amount was found to antagonize the vasoconstrictor action of adrenaline too, although not so completely; vasoconstriction could still be produced in response to a large (2 μg.) dose of adrenaline at a time when the effect of even a 1 mg. dose of methyl *iso*thiourea was quite suppressed. Sensitivity to methyl *iso*thiourea was very slowly restored when ordinary Ringer-Locke solution was perfused again in place of the anthisan solution.

Isolated gut

Slight but definite antagonism between anthisan and methyl *iso*thiourea was also noticed in organ bath experiments with guinea-pig ileum. Methyl *iso*thiourea tends to increase the tonus of the strip when it is added to make a bath concentration of M/10,000 or higher. This effect was found to be suppressed more or less completely when anthisan was given before the *iso*thiourea in concentrations of M/10,000 and upwards.

The tonus-increasing action of methyl *iso*thiourea on isolated rabbit ileum was not much impaired by a concentration of atropine (M/10,000) sufficient to render the muscle insensitive to even large amounts of acetylcholine. When guinea-pig ileum was used in place of rabbit ileum, however, it was found that this same concentration of atropine greatly reduced the response to a test dose of methyl *iso*thiourea.

In the presence of methyl *iso*thiourea the response of guinea-pig ileum to a test dose of histamine may be increased or decreased, according to the conditions employed. A decrease was always obtained when the dose of *iso*thiourea was of itself sufficient to produce a considerable rise in tone. Higher homologues like *n*-butyl and *n*-heptyl *iso*thiourea were found to exert a much stronger spasmolytic action, dose for dose. A slightly increased response to a small dose of histamine was seen occasionally with slightly smaller concentrations of methyl *iso*thiourea (of the order of M/10,000). Potentiation of the response to histamine by methyl *iso*thiourea was observed quite regularly when the strip had first been rendered moderately insensitive to histamine by treatment with anthisan. In these circumstances, doses of methyl *iso*thiourea or of *asym*-dimethylguanidine which had little effect themselves on muscle tone increased appreciably the response to a test dose of histamine.

TABLE I

EFFECTS OF *iso*THIOUREAS UPON THE BRONCHOCONSTRICTOR RESPONSE TO HISTAMINE

Substance	Dose	No. of exps. in which histamine response was		
		Increased	Unchanged	Decreased
Methyl *iso*thiourea	0.1 ml. M/10	5	4	
,, ,, ...	0.25 ml. ,,	4	4	
n-Hexyl ,, ...	0.1 ml. ,,			3
,, ,, ...	0.25 ml. ,,			1
n-Heptyl ,, ...	0.1 ml. ,,		1	2
,, ,, ...	0.25 ml. ,,			3
Tetramethylene di-*iso*thiourea ...	0.1 ml. M/20	1	2	
,, ,, ...	0.25 ml. ,,		1	2

Guinea-pig lung preparation

Slight transitory bronchoconstrictor responses were produced in the following experiments by injecting small test doses of histamine acid phosphate (2–10 μg./kg.) at regular intervals of five minutes or more. The response to histamine was altered by various *iso*thioureas as indicated in Table I. In about half the experiments performed with methyl *iso*thiourea the bronchoconstrictor effect of histamine was definitely potentiated (Fig. 3); it was never reduced. However, higher

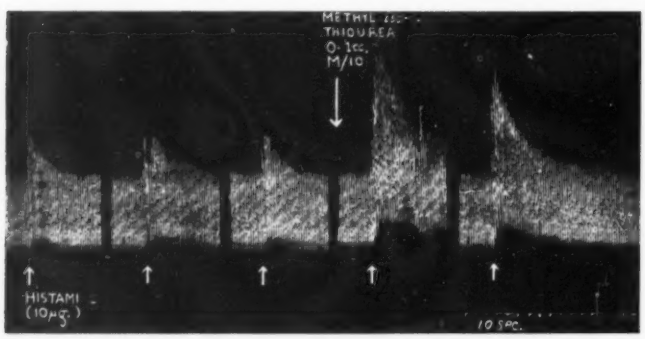

FIG. 3.—Guinea-pig lung preparation. The bronchoconstrictor effects were produced by 10 μg. doses of histamine acid phosphate injected at 10 min. intervals. The response to histamine is increased after a dose of methyl *iso*thiourea (0.1 ml. of an M/10 aqueous solution of the sulphate).

homologues usually produced anti-histamine effects when given in the same range of doses. Sometimes the effect of histamine was reduced by as much as 60–80 per cent.

Methyl *iso*thiourea had no distinctive effect *per se*; exceptionally it caused slight bronchoconstriction. In two of the experiments with *n*-heptyl *iso*thiourea there was considerable bronchoconstriction after its injection. Unfortunately the interpretation of some of these results was made difficult because spontaneous respiratory movements were resumed soon after the *iso*thiourea was injected. In most instances the compounds had no direct effect on the bronchioles.

Isolated rat uterus

It was confirmed that histamine has an inhibitory action on non-pregnant rat uterus. It should be added, however, that doses of the order of 1–5 μg./ml. were required to demonstrate any effect, although the horns were quite sensitive to adrenaline and acetylcholine.

Methyl *iso*thiourea was tested several times on each of eight preparations in concentrations of from M/10,000 to M/2,000. Usually it had no effect at all; sometimes there appeared to be slight stimulation. More definite evidence of stimulation was obtained with *n*-butyl *iso*thiourea. In four of six experiments a bath concentration of M/2,000 caused a slight increase in the tone and motility of the strip.

Effects on striped muscle preparations

Isolated frog rectus abdominis muscle

In ten successive experiments the addition of methyl *iso*thiourea (M/500) to the test dose of acetylcholine resulted in an enhanced response of the muscle. The *iso*thiourea alone did not increase muscle tone in four control experiments, but its potentiating activity became manifest in these too when the Ringer solution containing methyl *iso*thiourea sulphate was replaced by the test solution of acetylcholine.

Potentiation of the response to acetylcholine was also observed regularly in experiments with ethyl *iso*thiourea and with *asym*-dimethylguanidine. On the other hand, the long-chain amidine derivative *n*-hexyl *iso*thiourea was found to antagonize the action of acetylcholine even in a dilution of M/5,000.

Rat phrenic nerve-diaphragm preparation

An enhanced response to a short-acting (0.5 msec.) electrical stimulus—a slightly supermaximal shock given indirectly every 10 seconds—was obtained regularly after the injection of an M/10 solution of one of the following salts into the 100 ml. bath: guanidine hydrochloride (0.5–2.0 ml.), methylguanidine sulphate (0.5–1.0 ml.), methyl *iso*thiourea hydrochloride and sulphate (0.25–1.0 ml.), and ethyl *iso*thiourea hydrobromide (0.25–0.5 ml.). The phenomenon was seen almost immediately after the addition of methyl or ethyl *iso*thiourea, but not for some minutes after the addition of guanidine or of *asym*-dimethylguanidine.

Potentiation after methyl *iso*thiourea was still observed in experiments in which a longer-acting electrical stimulus (35 msec.—the maximum for the instrument) was applied or in which the rate of stimulation was ten times faster.

The higher homologues *n*-butyl and *n*-hexyl *iso*thiourea reduced the amplitude of the twitch when added in the same range of doses as methyl *iso*thiourea. Transitional behaviour was displayed by *n*-propyl *iso*thiourea: whereas moderate concentrations (M/2,000–M/500) somewhat increased the amplitude of the twitch, higher concentrations decreased it.

Interaction with d-tubocurarine.—A dose of 1 μg./ml. of *d*-tubocurarine chloride was found sufficient to curarize the preparation ; the muscle could now be made to contract only by stimulating it directly. Given at this point guanidine (M/500) increased considerably the contractions brought about by direct stimulation, but indirect stimulation indicated that this potentiating effect of guanidine was due in part to an anti-curare action ; such an action was demonstrated directly in six experiments. Methyl *iso*thiourea was found to behave similarly when given in a concentration of M/4,000. Its potentiating action was seen best when the dose of *d*-tubocurarine administered was one which produced only partial block.

When the dose of *d*-tubocurarine was increased to 2 μg./ml., no anti-curare action could be demonstrated with the above doses of methyl *iso*thiourea and guanidine. Nevertheless, both amidine derivatives tended to augment the effect of direct electrical stimuli if given under these conditions ; methyl *iso*thiourea to nearly its usual extent but guanidine sometimes hardly at all (Fig. 4).

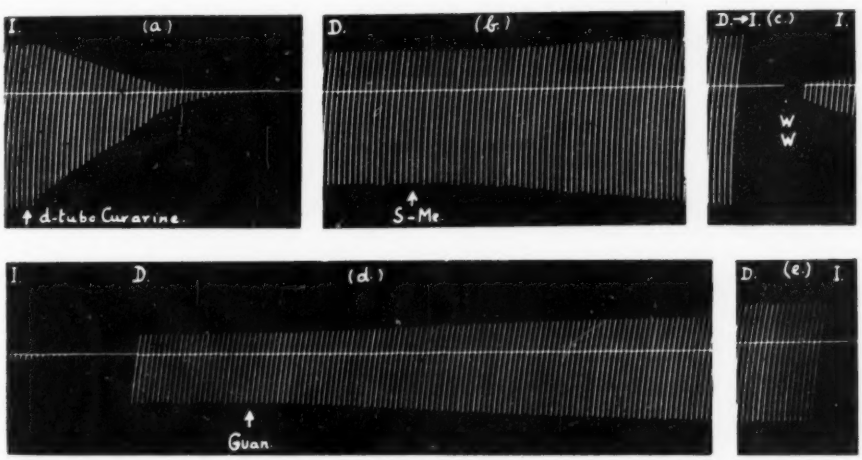

FIG. 4.—Rat diaphragm preparations. I indicates indirect and D direct stimulation. (*a*) The effect of maximal indirect shocks (1.7 m.amp., 0.5 msec. shocks at 10 sec. intervals) is abolished by a 200 μg. dose of *d*-tubocurarine. (*b*) Same preparation. Contractions now elicited by direct electrical stimulation. Methyl *iso*thiourea hydrochloride (M/4,000 at the arrow) enhances the response to these, although (*c*) indirect stimulation shows that the preparation remains fully curarized. After two washings, sensitivity to indirect stimuli slowly returns. (*d, e*) A similar experiment with guanidine hydrochloride (M/500 at the arrow). There is a 10 min. interval between strips (*d*) and (*e*).

Influence of calcium ion concentration. — A reduction in the effective concentration of calcium ions in the bath was brought about (i) by adding sodium oxalate solution to the bath, (ii) by adding sodium citrate solution, or (iii) by replacing the ordinary Ringer-Tyrode solution in the bath with a solution containing less than the normal amount of calcium chloride. It was noted, confirming McDowall (1949), that a moderate reduction in the calcium ion concentration of the bath usually increases the muscle twitch, whereas a large reduction invariably decreases it. In the latter instance, adding calcium chloride solution restored the amplitude of the twitch ; the normal effect of an increase in calcium ion concentration was to reduce the twitch.

In six experiments guanidine (M/500) was added to Ringer-Tyrode solution containing half the normal amount of calcium. The average increase in the size of the maximal twitches in these six experiments was definitely less than that observed in seven control experiments in which the same dose of guanidine was added to normal Ringer-Tyrode solution. Raising the calcium content of the bath was less effective for counteracting the action of guanidine. One of the most striking features of this set of experiments was the persistence of the guanidine effect. Even ten or fifteen washings failed to restore the normal reaction of the preparation. Consequently little could be learned from the effect of a second dose.

Similar experiments were carried out with methyl *iso*thiourea. As a test dose, 0.5 ml. of an aqueous M/10 solution was injected into the 100 ml. bath. In ordinary Tyrode solution this dose of methyl *iso*thiourea caused a slow steady increase in the size of the twitch in almost all of some twenty trials. The response to the *iso*thiourea was reduced —sometimes quite suppressed—when the calcium ion concentration was lowered sufficiently to reduce the amplitude of the twitch ; but it was not much affected by doubling or quadrupling the calcium content of the bath.

Interaction with potassium.—Raising the potassium ion concentration of the Ringer-Tyrode solution in the bath at first potentiates increas-

ingly the response to a maximal indirect shock, then reduces it—just as does a typical amidine derivative when it is given in increasing amount.

It was observed that after two 20 mg. doses of potassium chloride (at which time a further increase in the potassium ion concentration of the 100 ml. bath could no longer increase the size of the twitch), test doses of methyl or ethyl *iso*thiourea were unable to cause potentiation. Conversely, a test dose of potassium chloride given after a large dose of a short-chain amidine derivative produced much the same sort of effect as would be expected if excess potassium were already present. In experiments with guanidine this change in the reaction of the preparation was still seen after eight or ten washings—which is in accord with the persistence of other effects of guanidine. Amongst alkyl *iso*thioureas, the *n*-hexyl derivative was found to antagonize the potentiating effect of potassium chloride more effectively than those with shorter side-chains.

Cat sciatic nerve-gastrocnemius preparation

Methyl *iso*thiourea sulphate was tested in doses of 2–5 mg./kg. on eleven cats. Almost as soon as the drug solution entered the iliac artery the " maximal " contractions produced by stimulating the muscle through the sciatic nerve six or twelve times a minute were increased, usually by some 10–20 per cent (Fig. 5*c*). When given in the same range of doses guanidine hydrochloride had practically no effect, while *n*-hexyl *iso*thiourea hydrobromide decreased the height of the contractions.

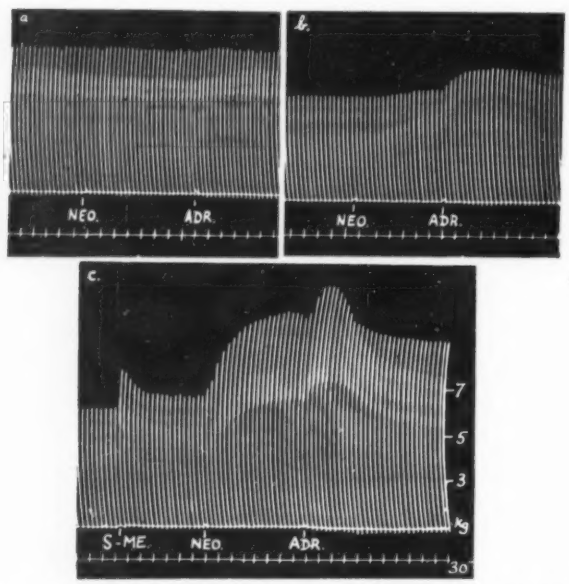

FIG. 5.—Cat ♀, 2.8 kg., under chloralose. Sciatic nerve-gastrocnemius muscle preparation. Contractions elicited by maximal shocks at 10 sec. intervals. Neostigmine given at 90 min. intervals (doses of 10 μg. at NEO) followed 2–5 min. later by adrenaline (10 μg. at ADR). Note the potentiated response to neostigmine after the administration of methyl *iso*thiourea sulphate 10 mg. at S-Me).

If muscle contractions were first decreased some 20–30 per cent by giving a small dose of *d*-tubocurarine, methyl *iso*thiourea caused more striking potentiation than usual. If, however, enough *d*-tubocurarine was given to produce an 80–90 per cent decrease, the effect of a subsequent dose of methyl *iso*thiourea was relatively slight.

In eight experiments small doses of neostigmine (3–6 μg./kg.) were given every 90 minutes. Even this long interval between doses was usually insufficient to prevent cumulation entirely. However, cumulation alone seemed inadequate to explain the greatly enhanced effect of a test dose of neostigmine given after either methyl *iso*thiourea or guanidine (Fig. 5). In three of the four experiments made with each compound it appeared certain that the amidine derivative had potentiated the effect of the neostigmine.

Adrenaline had little or no effect on this preparation when given in a dose of 3–5 mg./kg., but after the administration of neostigmine the test dose of adrenaline usually produced a considerable increase in the size of the muscle twitches. No corresponding effect was seen in parallel experiments with guanidine or methyl *iso*thiourea. However, in several preparations in which the adrenaline caused only a decrease in the size of the contractions, this depressant effect was seen to be enhanced after methyl *iso*thiourea had been given.

DISCUSSION

In their effects upon smooth muscle preparations amidine derivatives like methyl *iso*thiourea and guanidine resemble histamine rather than adrenaline. This is shown especially by the experiments with blocking agents. Whereas the vasconstrictor action of methyl *iso*thiourea on the rabbit's ear preparation was antagonized appreciably by " Anthisan " (an anti-histamine drug), it was hardly affected by " Priscol " (which is predominantly an anti-adrenaline). Not all actions of histamine on muscle are reproduced, however. The amidine derivatives tested showed no tendency to relax rat uterus. They differed from histamine moreover in acting on striped muscle almost as strongly as on smooth.

When effects on striped muscle preparations are taken into consideration it becomes evident that

these amidine derivatives resemble potassium more closely than histamine: like potassium, they potentiate the effect of "maximal" electrical shocks delivered to rat diaphragm muscle through the phrenic nerve; they also antagonize the effect of low concentrations of *d*-tubocurarine on the rat diaphragm preparation whilst having their own effects reduced by higher concentrations of *d*-tubocurarine—which suggests that they compete with it for the same receptors (cf. Quilliam and Taylor, 1947). Moreover, potassium salts in moderate excess resemble typical amidine derivatives in causing the contraction of most types of smooth muscle; they also have a vasoconstrictor action which is not affected appreciably by the treatment with ergotoxine, apart from one caused by the liberation of adrenaline (Mathison, 1911; Knoefel and Alles, 1938; Fastier and Smirk, 1947; Sugawara and Tada, 1927).

It must be added that the majority of these effects can also be reproduced by a reduction in calcium ion concentration. Thus in comparing the effects of guanidine on frog rectus muscle with those of calcium deprivation, Harvey (1940) observed that "both bring about a sensitization to potassium ions, their action is antagonized by excess of calcium, and both lead to a 'spontaneous' activity." The close correspondence between symptoms of guanidine poisoning and those of parathyroid tetany has long been known (Paton and Findlay, 1917).

Nevertheless, so closely linked are the physiological actions of potassium and calcium that Harvey and others have been content to attribute the pharmacological effects of guanidine to changes in ionic balance rather than to anything so specific as calcium lack or potassium excess. Neither possibility is ruled out by the above results, but it seems significant that the chemical relatives of methyl *iso*thiourea which have been found to resemble it at all closely in pharmacological properties are those whose salts ionize freely to yield small cations (Fastier, 1948). In order to explain the effects of these various bases in terms of "potassium excess" one need only assume that they can simulate more or less closely the action of potassium ions, unlike their higher homologues, which show a far greater tendency to produce only inhibitory effects (Fastier and Reid, 1948). It is far less easy to explain how a reduction in the effective concentration of calcium ions might be brought about.

Whatever be the precise explanation, it is obvious that amidine derivatives have a deep-seated action on muscle. A diversity of muscle-contracting agents have their effects modified in the presence of amidine derivatives. Antihistamine effects on gut and bronchioles have been observed with large doses of amidine derivatives and potentiation of histamine effects with smaller doses. Analogous effects upon sensitivity to the vasoconstrictor action of adrenaline have been described by Fastier and Reid (1948). Pharmacological effects of acetylcholine too may be modified by typical amidine derivatives.

It has been known for some considerable time that guanidine can potentiate the action of acetylcholine on striped muscle (Frank, Stern, and Nothmann, 1921; Harvey, 1940). The finding that bases like *asym*-dimethylguanidine, methyl and ethyl *iso*thiourea also possess this property is hardly surprising considering the numerous other analogies that have been noted in their pharmacological behaviour (Fastier and Smirk, 1947). That guanidine can sensitize frog rectus muscle to potassum as well as to acetylcholine has been shown by Harvey (1940). The ability of guanidine to potentiate the effects of a variety of muscle-contracting agents is further evident from results of Camis (1909), Paton and Findlay (1917), Führer (1920), and Kato (1939) on striped muscle, and of Führer (1917), Burns and Watson (1920), Godeaux (1942), and García and Perdomo (1946) on smooth muscle preparations. In the rat phrenic nerve-diaphragm preparation the increased response to "maximal" electrical shocks applied indirectly may well depend solely upon an enhanced sensitivity to the acetylcholine liberated at myoneural junctions. However, this leaves unexplained the observation that some potentiation can still be obtained after curarization.

Guanidine has been found to be of some value for the treatment of myasthenia gravis (Minot, Dodd, and Riven, 1939), though why it affords relief to myasthenics is far from evident. Guanidine does not inhibit cholinesterase (Minot, 1939; Thompson and Tice, 1941). Therefore it might be concluded that the action of this drug is quite different from that of neostigmine; but Thompson and Tice question such a view. They point out *inter alia* that the duration of relief of symptoms in myasthenia gravis after neostigmine follows the change in serum potassium more closely than it does the decrease in activity of cholinesterase. Like Cumings (1939), they believe that some abnormality of potassium metabolism is present in myasthenia gravis. The above results suggest that potentiation of the effect of acetylcholine is but one manifestation of a more general action of guanidine on striped muscle, and possibly it is

not this but some related effect which is of therapeutic value in myasthenia gravis. It may be remarked in conclusion that, as several other amidine derivatives have been shown to resemble guanidine closely in their effects upon striped muscle preparations, some of them might also prove of clinical value.

Summary

1. Effects of methyl *iso*thiourea and guanidine salts have been studied on perfused rabbit ears, isolated strips of rabbit and guinea-pig ileum, isolated rat uterus, guinea-pig bronchioles (Konzett-Rössler technique), isolated frog rectus muscle, cat sciatic nerve-gastrocnemius, and rat phrenic nerve-diaphragm preparations. A few experiments have also been performed with salts of other alkyl *iso*thioureas and of methyl- and *asym*-dimethylguanidine.

2. The short-chain amidine derivatives tend to increase the tonus of smooth muscle, though their effect on some preparations is negligible. The vasconstrictor response to methyl *iso*thiourea, unlike that of adrenaline, is not antagonized appreciably by "Priscol," but both the vasoconstrictor action of methyl *iso*thiourea and its tonus-increasing action on gut are partially antagonized by the anti-histamine drug "Anthisan."

3. The effects of methyl *iso*thiourea and of guanidine on striped muscle preparations resemble those of potassium in excess. They are able to potentiate the effect of maximal electrical shocks applied indirectly to the rat diaphragm preparation; they exert an appreciable anti-curare action; and their potentiating action on the rat diaphragm preparation is not readily antagonized by *d*-tubocurarine.

4. Several of the short-chain amidine derivatives have been shown to enhance the response of frog rectus muscle to test doses of acetylcholine. Potentiation of effects of histamine on gut and bronchioles has also been observed under conditions described in the text. References to yet other potentiating actions of amidine derivatives on muscle are given.

5. The mode of action of these compounds is discussed, particularly in relation to that of histamine and of potassium.

It is a pleasure to acknowledge here my indebtedness to Professor J. H. Burn, who has afforded me every facility for carrying out this work and has constantly guided its progress.

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SOME RELATIONSHIPS BETWEEN ANTI-NICOTINE ACTIVITY AND SPECIFIC ANTAGONISMS

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Besides atropine, new synthetic compounds with parasympathicolytic and spasmolytic activity have been shown recently to possess therapeutic value in Parkinson's disease; these are diethylaminoethyl-1-phenyl-*cyclo*pentane-1-carboxylate (DPCPC) (Grünthal, 1946; Hartmann, 1946; Schwab and Leigh, 1949) and according to Sigwald *et al.* (1946) and Bovet *et al.* (1947, 1948) N-diethylaminoethylphenothiazine (DPT). The first is parasympathicolytic and papaverine-like (Domenjoz, 1946; Frommel *et al.*, 1949) and exhibits at the same time some antihistamine (Frommel *et al.*, 1949), peripheral curare-like (Domenjoz, 1946), and local anaesthetic (Fleisch and Baud, 1948) properties. In addition to these effects, Heymans *et al.* (1948a and b, 1949b and c) have demonstrated that DPCPC is a powerful anti-nicotine agent because it suppresses all the toxic effects of high doses of this alkaloid. The same authors have recently shown that DPT also exerts a strong anti-nicotine activity in protecting dogs against 100–200 lethal doses of nicotine (1949a, b, and c).

The very complex pharmacological activity of nicotine is generally divided into peripheral effects on muscular fibres and neuromuscular junctions, effects on autonomic ganglia, and effects on the central nervous system. Further and at each level, the action of nicotine depends on the alkaloid concentration and on the degree of intoxication, so that these can also be classified into motor and inhibitory effects (Bovet *et al.*, 1948; Goodman and Gilman, 1946). Corresponding to these modes and sites of action, nicotine can thus produce some effects on synaptic activity which, according to Langley and Dickinson (1889), can be used as a test of nicotine-like or anti-nicotine activity. On the other hand many other effects such as those on the respiratory, cardiovascular or central nervous system, or on isolated organs—e.g., intestine, vessels, or heart—do not show a clear relationship to the usual physiological stimulations. As it was supposed that the therapeutic activity of some compounds in Parkinson's disease might be related to their ganglionic-blocking effect on the tonic centres of the extrapyramidal tracts or on the cholinergic transmission (Sigwald *et al.*, 1946; Bovet *et al.*, 1947, 1948), we thought that it would be interesting to investigate more closely the relationship between anti-nicotine effect and parasympathicolytic or autonomic activity. For a better discrimination of such antagonisms, it seemed also necessary to extend our investigations to compounds of diverse chemical constitution possessing some specific activity such as antagonism of parasympathetic or sympathetic effects, spasmolysis, ganglionic-blocking, local anaesthesia, and even central depression.

We tested at first the overall antagonism against nicotine on the whole animal by determining protective indices.

METHOD

Mice weighing about 20 g. received intravenously a toxic dose of nicotine (0.00125 g./kg.) which produced convulsions, paralysis, and death in all animals. One hour previously, doses of the presumed protective agent were given subcutaneously to groups of 6–10 mice, and the ED50, protecting 50 per cent of animals, was determined according to Miller and Tainter's method (1944) which allows if necessary a statistical treatment of the results.

In a first series of experiments we tested the anti-nicotine effect of atropine as a true parasympathicolytic agent compared with DPCPC and DPT and of two synthetic compounds with neurogenic and myogenic activity (Meier, 1936; Johnson and Reynolds, 1937; Meier and Hoffmann, 1940; Graham and Lazarus, 1940; Tripod, 1949)—viz., "Trasentin" (diethylaminoethylester of diphenylacetic acid) and "Trasentin-H" (diethylaminoethylester of phenyl-*cyclo*hexylacetic acid). A specific sympathicolytic agent like ergotamine, and tetraethylammonium

bromide (TEA), which shows some anti-nicotine effect (Boelaert, 1948) besides its marked ganglionic-blocking action (Acheson *et al.*, 1945, 1946a and b; Trendelenburg, 1923), were also investigated. Procaine and cocaine were tested because the former possesses, if not a pure ganglion-blocking effect (Bovet *et al.*, 1948), at least some parasympathicolytic and anti-nicotine properties (Hazard *et al.*, 1942a and b; Moore *et al.*, 1948; Haimovici, 1948; Soehring and Hessler, 1949). Finally *d*-tubocurarine as a nerve-muscle blocking agent (Feldberg and Lin, 1949) and phenobarbitone as a general depressant of the central nervous system were used.

In a second series of determinations we tested in the same manner the anti-acetylcholine effect of all these compounds against an intravenous dose of acetylcholine (0.020 g./kg.) which produces convulsions, paralysis, and death in all animals.

Results

Table I gives the ED50 values for the anti-nicotine and anti-acetylcholine activities of all these agents.

TABLE I

ANTI-NICOTINE AND ANTI-ACETYLCHOLINE ACTIVITY OF VARIOUS AGENTS ON MICE

Class of drug	Drug	Anti-nicotine ED50 g./kg.	Anti-acetylcholine ED50 g./kg.
Parasympathicolytic and spasmolytic	Atropine	<0.800	0.009
	DPT	0.014	0.130
	DPCPC	0.031	0.013
	Trasentin	0.031	0.035
	Trasentin-H	0.085	0.018
Sympathicolytic	Ergotamine	>0.100	>0.100
Ganglion-blocking	Tetraethyl-ammonium	0.028	>0.200
Local anaesthetic	Cocaine	0.017	>0.200
	Procaine	0.100	>0.400
Peripheral-blocking	*d*-Tubocurarine	>0.00075	>0.00075
Central ...	Phenobarbitone	0.007	>0.100

These results show clearly that the general toxicity of nicotine can be antagonized by spasmolytic and ganglion-blocking agents or even by cocaine as well as by a central depressant. These findings seem thus to be related not only to a specific effect but also to various sites of action of the alkaloid. In addition, the lack of activity of atropine is very striking so that a parasympathomimetic effect of nicotine is unlikely. Further, this test also clearly demonstrates that a good anti-nicotine activity is not exclusively exhibited by DPT and DPCPC, since trasentin and to some extent trasentin-H can also antagonize the effects of nicotine.

On the other hand, an anti-acetylcholine activity is shown only by atropine and by spasmolytic agents which are known to possess parasympathicolytic properties like DPT (Bovet *et al.*, 1947, 1948; Gordon, 1948; Heymans *et al.*, 1949a, b, and c), DPCPC (Domenjoz, 1946; Bovet *et al*, 1948; Heymans *et al.*, 1948a and b; Frommel *et al.*, 1949), trasentin, and trasentin-H (Meier, 1936; Meier and Hoffmann, 1940; Graham and Lazarus, 1940; Bovet *et al.*, 1948; Tripod, 1949).

TABLE II

RELATIONSHIP BETWEEN ANTI-NICOTINE AND ANTI-ACETYLCHOLINE ACTIVITIES

Drug	Index $\frac{\text{anti-nicotine potency}}{\text{anti-acetylcholine potency}}$
Atropine	<0.01
DPT	9.30
DPCPC	0.42
Trasentin	1.13
Trasentin-H ...	0.21

The ratios between the ED50 values for the anti-nicotine and anti-acetylcholine activities (Table II) demonstrate that these properties are clearly independent in these spasmolytic compounds.

Since an anti-nicotine activity on the whole animal is exhibited by various compounds, which are neither chemically nor pharmacologically related, it seemed necessary to compare the effect of nicotine with typical autonomic effects in a simpler test.

Acetylcholine has a consistently stimulant effect on isolated rabbit and guinea-pig ileum. Nicotine on the contrary produces on the intestine a contraction as described by Trendelenburg (1917), Alvarez (1918a and b), von Oettingen *et al.* (1928), and recently by Feldberg and Lin (1949), or an inhibition of the activity with higher concentrations according to Langley and Magnus (1905). This effect can be mixed or biphasic (Alvarez, 1918), and depends on the dose, the anatomical localization of the intestinal strip, the animal species, as well as on the experimental technique (Alvarez, 1918; Raymond-Hamet, 1930; Bovet

FIG.

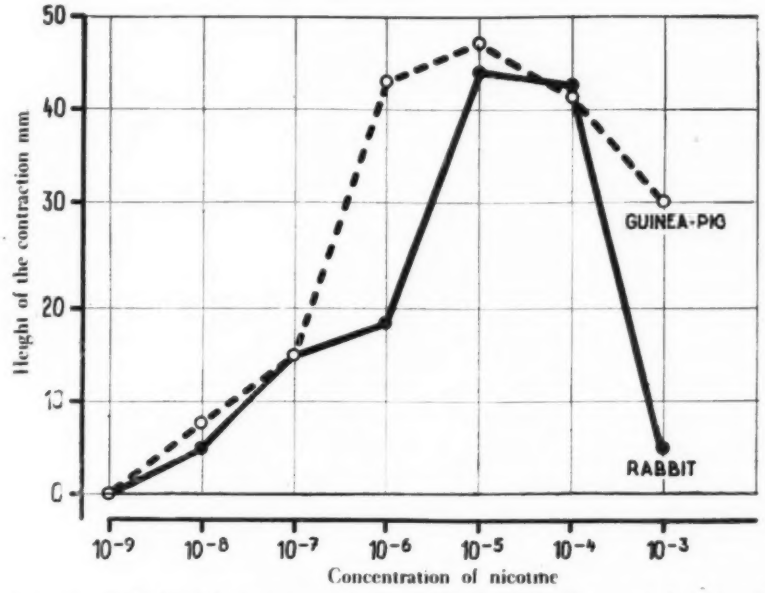

FIG. 1.—Relationship between concentration of nicotine and height of contraction of the isolated guinea-pig and rabbit ileum.

et al., 1948). It seemed therefore necessary to establish a test in which a typical motor effect of nicotine would be produced.

METHOD

Isolated strips of rabbit or guinea-pig ileum were kept at 0° during four hours and afterwards suspended in a 50 c.c. bath containing oxygenated Tyrode. The bath temperature was 38° C. and a frontal writing lever was used. The concentrations mentioned in the text are the final concentrations in the bath.

RESULTS

The mean response-dose curves for nicotine on the rabbit and guinea-pig ileum are depicted in Fig. 1.

Guinea-pig ileum seemed suitable for the testing of a nicotine antagonism because its motor effect has a wider range; moreover, a biphasic response is only elicited by concentrations higher than 10^{-4}, and the contractions can be reproduced quite regularly without tachyphylaxis, at least with low concentrations as already described by Feldberg and Lin (1949). Rabbit ileum, on the other hand, reacts more irregularly to the alkaloid and shows even in low concentrations, for example 10^{-7}, a biphasic or polyphasic effect.

The antagonistic activities of various agents were tested on isolated guinea-pig ileum against a final nicotine concentration of 10^{-6}. The anti-nicotine effects recorded after the prophylactic treatment with the antagonist could then be measured and the inhibition statistically treated for "all or none" (Morrell *et al.*, 1940; Miller *et al.*, 1948) or graded responses (Schild, 1942; Holton, 1948; Tripod, 1949). Figs. 2 and 3 illustrate the antagonistic effects of atropine and trasentin-H. The values of the concentrations producing a 50 per cent anti-nicotine effect (ED50)

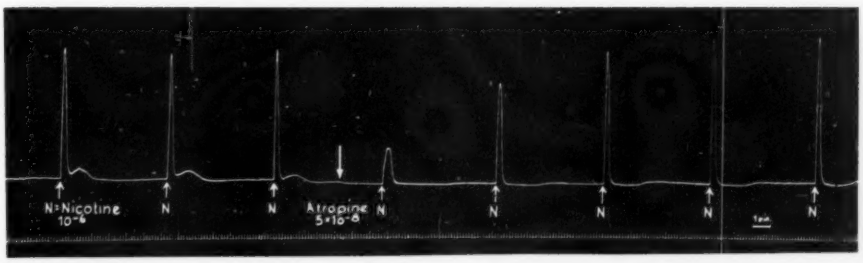

FIG. 2.—The anti-nicotine effect of 5×10^{-8} atropine is illustrated on isolated guinea-pig ileum.

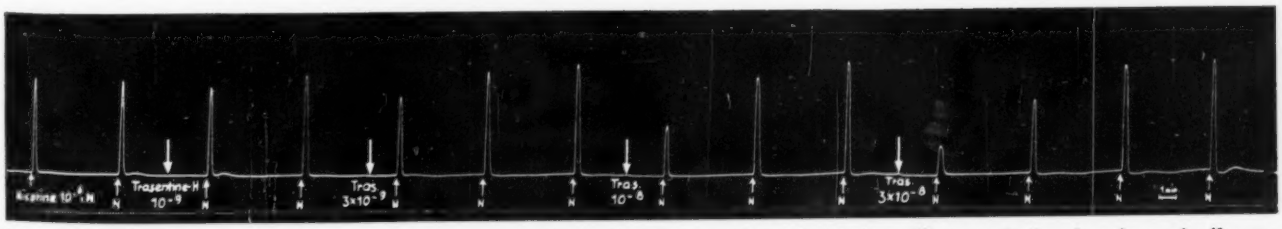

FIG. 3.—Trasentin-H produces even in a concentration of 10^{-9} a small anti-nicotine effect on isolated guinea-pig ileum. The inhibition of the motor effect of nicotine is marked with 3×10^{-8}.

TABLE III

ANTI-NICOTINE ACTIVITY OF VARIOUS AGENTS ON ISOLATED GUINEA-PIG ILEUM

Class of drug	Drug	Anti-nicotine ED50	Relative potency
Parasympathicolytic and spasmolytic	Atropine	2×10^{-8}	100
	DPT	1×10^{-7}	20
	DPCPC	2×10^{-8}	100
	Trasentin	6×10^{-8}	33
	Trasentin-H	2×10^{-8}	100
Sympathicolytic	Ergotamine	10^{-5}	0.2
	Priscol	10^{-5}	0.2
Ganglion-blocking	Tetraethylammonium	$>10^{-4}$	<0.02
Local anaesthetic	Cocaine	$>10^{-4}$	<0.02
	Procaine	10^{-5}	0.2
Peripheral-blocking	*d*-Tubocurarine	3×10^{-4}	0.006
Central ...	Barbitone	$>10^{-4}$	<0.02
	Phenobarbitone	10^{-4}	0.02

have been graphically evaluated from several experiments and are listed in Table III, where the relative potency of atropine is put as 100.

The greatest anti-nicotine activity is strikingly shown by atropine and the spasmolytic compounds, whereas sympathicolytic, ganglion-blocking, local anaesthetic, and peripheral-blocking agents as well as central depressants all show an antagonism, but in such high concentrations that their specificity is rather doubtful. In their recent paper Feldberg and Lin (1949) drew attention to the anti-nicotine effect of local anaesthetics and of *d*-tubocurarine on the guinea-pig and the rabbit ileum; in our experiments on guinea-pig gut, however, this inhibition was found to be much smaller than that of atropine.

In the same manner we tested on the rabbit ileum the antagonism of some agents against a final concentration of acetylcholine of 5×10^{-7}. The ED50 values are compiled in Table IV, where the relative potency of atropine is again 100.

As expected, only atropine and atropine-like agents are here effective, but the spasmolytic compounds possess different potencies for anti-nicotine and anti-acetylcholine activity as shown by Tables III and IV, although both are produced by rather similar concentrations.

Further, we compared various antagonistic effects, in order to establish the relationships between the anti-nicotine specificity, the sympathicolytic activity against 3×10^{-6} adrenaline on the seminal vesicle of the guinea-pig (Brügger, 1945), and the antagonism against 2×10^{-4} $BaCl_2$ on the rabbit ileum.

TABLE IV

ANTI-ACETYLCHOLINE ACTIVITY OF VARIOUS AGENTS ON ISOLATED RABBIT ILEUM

Class of drug	Drug	Anti-acetylcholine ED50	Relative potency
Parasympathicolytic and spasmolytic	Atropine	2.3×10^{-8}	100
	DPT	4×10^{-7}	5.7
	DPCPC	2×10^{-7}	11.5
	Trasentin	4.4×10^{-7}	5.2
	Trasentin-H	1.6×10^{-7}	14.4
Ganglion-blocking	Tetraethylammonium	3.2×10^{-4}	0.007
Local anaesthetic	Cocaine	2.3×10^{-4}	0.01
	Procaine	2.1×10^{-5}	0.11
Peripheral-blocking	*d*-Tubocurarine	$>10^{-4}$	<0.02
Central ...	Phenobarbitone	$>10^{-4}$	<0.02

TABLE V

RELATIONSHIP BETWEEN ANTI-NICOTINE EFFECT AND OTHER ANTAGONISMS ON ISOLATED ORGANS

Drug	Indices of specific potencies		
	Anti-nicotine / Anti-acetylcholine	Anti-nicotine / Sympathicolytic	Anti-nicotine / Musculotropic
Atropine ...	1.1	3,000	50,000
DPT ...	4	30	100
DPCPC ...	10	1,000	500
Trasentin	7.3	333	167
Trasentin-H	8	1,000	150

Calculated ratios of anti-nicotine activities to other antagonistic activities are reproduced in Table V. They show that the anti-nicotine activity is more closely related to the anti-acetylcholine activity than to any of the other antagonisms.

On the isolated guinea-pig ileum it can thus easily be demonstrated that atropine and spasmolytic compounds like DPT, DPCPC, trasentin, and trasentin-H exert a specific antagonism against the nicotinic stimulation, antagonism which is not related to anti-adrenaline or musculotropic properties. A closer relationship is only exhibited with

an anti-acetylcholine effect, so that this point seemed worthy of further investigation. Therefore, we tested the antagonistic effect of these agents against other substances with muscarine-like properties such as arecoline and pilocarpine (Bovet *et al.*, 1948) and determined the various antagonistic ED50 values on the guinea-pig ileum against acetylcholine (10^{-7}), arecoline (3×10^{-8}), and pilocarpine (5×10^{-7}) (Tables VI and VII).

TABLE VI

ANTAGONISMS ON ISOLATED GUINEA-PIG ILEUM

Drug	Anti-acetyl-choline ED50	Anti-nico-tine ED50	Anti-areco-line ED50	Anti-pilo-carpine ED50
Atropine ...	7×10^{-9}	2×10^{-8}	7×10^{-10}	2×10^{-9}
DPT ...	2×10^{-7}	10^{-7}	2×10^{-8}	4×10^{-8}
DPCPC ...	8×10^{-8}	2×10^{-8}	2×10^{-8}	3×10^{-8}
Trasentin ...	4×10^{-7}	6×10^{-8}	3×10^{-8}	6×10^{-8}
Trasentin-H	2×10^{-8}	2×10^{-8}	2×10^{-9}	3×10^{-9}

TABLE VII

RELATIONSHIP BETWEEN ANTI-NICOTINE EFFECT AND OTHER ANTAGONISMS ON ISOLATED GUINEA-PIG ILEUM

Drug	Indices of specific potencies		
	Anti-nicotine / Anti-acetylcholine	Anti-nicotine / Anti-arecoline	Anti-nicotine / Anti-pilocarpine
Atropine ...	0.35	0.035	0.1
DPT ...	2.0	0.20	0.4
DPCPC ...	4.0	1.00	1.5
Trasentin ...	6.7	0.50	1.0
Trasentin-H	1.0	0.10	0.15

Similar experiments showed that tetraethylammonium, cocaine and procaine, as well as *d*-tubocurarine, exhibit only an unspecific antagonism against these muscarine-like substances in concentrations higher than 10^{-5}.

Discussion

These findings show that anti-acetylcholine, anti-nicotine, anti-arecoline, and anti-pilocarpine properties are closely related but that, nevertheless, differences of specificity occur. In spite of this, it seems that the group of spasmolytics investigated all exhibit the same type of antagonism against a muscarine-like effect.

The site of action of nicotine on the isolated guinea-pig ileum has thus to be considered. Its motor effect could theoretically be due to a ganglionic excitation by low concentrations of the alkaloid. In our experiments, however, it was demonstrated that a specific ganglionic-blocking compound like tetraethylammonium cannot antagonize the stimulation produced by 10^{-6} nicotine (Table III). As our experiments were done on ice-treated ileum in order to obtain a greater and more regular sensitivity to nicotine, it might be objected that this treatment already "blocks" the autonomic ganglia of the intestine; this is not the case because we obtained the same values as those listed in Table III with the ileum of freshly killed guinea-pigs, with perhaps a little higher sensitivity to cocaine, procaine, and *d*-tubocurarine which, however, still remains 100–1,600 times smaller than for atropine. A usual ganglionic site of action being excluded, the motor effect of nicotine can be explained by special pharmacological properties of the intestinal ganglia of the guinea-pig, or by a peripheral action on the neural elements—e.g., a post-ganglionic stimulation. The analogy with arecoline and pilocarpine which also have a postganglionic and parasympathomimetic action (Bovet *et al.*, 1948) strengthens this hypothesis. Moreover, a post-ganglionic action of nicotine has already been demonstrated on the blood vessels by Haimovici (1948).

In any case, an anti-nicotine activity on the whole animal seems to be related to various mechanisms involved in the different sites of action of nicotine. This effect is not exclusively a property of spasmolytic compounds with therapeutic activity in Parkinson's disease like DPT and DPCPC, since trasentin and trasentin-H equally show a good anti-nicotine effect, and atropine does not show it at all. Furthermore, the anti-nicotine activity on isolated organs is comparable to an anti-acetylcholine or anti-muscarine mechanism, and it is again exhibited with high specificity not exclusively by spasmolytic compounds with therapeutic activity in Parkinson's disease. It seems thus that the therapeutic activity of DPT and DPCPC is produced by a different and characteristic mechanism, but neither the anti-acetylcholine nor the anti-nicotine mechanism shows a convincing parallelism with therapeutic activity in Parkinson's disease.

Summary

1. On mice, the toxicity of nicotine can be antagonized by spasmolytic and ganglionic-blocking agents as well as by a central depressant. These anti-nicotine properties seem to be related to various sites of action of the alkaloid and not to a specific blocking of parasympathetic effects.

2. On the isolated guinea-pig ileum nicotine produces a muscarine-like stimulation which can be antagonized by atropine and spasmolytic compounds. These anti-nicotine properties are related more to an anti-acetylcholine or anti-muscarine effect than to a sympathicolytic or musculotropic activity.

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THE TOLERANCE OF THE DOG TO INTRATHECAL INJECTIONS OF CRYSTALLINE PENICILLIN

BY

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Although it is known that intrathecal administration of penicillin may have an irritant action on the meninges and on the central nervous system (Walker, 1947 ; Cairns, 1947), no measurements of the degree of tolerance either in the human subject or in the experimental animal are yet available. Moreover penicillin is generally injected into the subarachnoid space dissolved in isotonic sodium chloride solution. Isotonic sodium chloride solution has itself a well-defined irritant action on the meninges (Bedford, 1946, 1948a, 1948b). It is therefore essential that this action should be taken into consideration when determining that of penicillin. In the experiments now reported, a study has been made of the effect of the intracisternal injection of crystalline penicillin in dogs.

Experimental Procedure

The general experimental procedure was similar to that described in earlier publications (Bedford, 1946, 1948a), to which reference for details should be made. In this series of experiments, the dogs were anaesthetized with ether. Crystalline sodium penicillin G(11) ("Avlon," Imperial Chemical Pharmaceuticals, Ltd.) was used throughout the experiments. The penicillin was dissolved in sterile distilled water immediately before injection into the cisterna magna ; in a few experiments it was dissolved in a 0.9 per cent (w/v) solution of sodium chloride in distilled water. Although solutions of penicillin in distilled water were found to have an average *p*H of 5.5, they were in no sense buffered at that *p*H. After the addition of a relatively small volume of cerebrospinal fluid, the solutions rapidly assumed the *p*H of that fluid. A constant volume (1.0 c.c.) of solution was introduced in all experiments. The animals after recovery from the anaesthetic were allowed to survive for six hours ; in a few experiments the survival time was 24 hours. Any animal that displayed symptoms of severe irritation of the nervous system after recovery from the anaesthetic was not allowed to survive.

Results

The effect of the intracisternal administration of 100,000 and 20,000 i.u. penicillin

Two dogs weighing 9.0 kg. and 8.5 kg. respectively were each given 100,000 i.u. penicillin dissolved in 1.0 c.c. distilled water and kept under observation while they recovered from the anaesthetic. As the effects of the ether wore off, the animals were found to be holding the head rigidly retracted ; they repeatedly licked the nose and attempted to scratch the back of the neck with the hind leg. There was a profuse flow of saliva. Generalized tremors heralded the onset of convulsions which speedily passed into status epilepticus. The symptoms displayed by both animals were almost identical. Similar effects were obtained after the administration of 20,000 i.u. to two dogs weighing 8.0 kg. and 8.5 kg. respectively. The animals were destroyed after the onset of general convulsions before complete consciousness had been regained.

The effect of the intracisternal administration of 10,000 i.u. penicillin

The experiments were divided into three groups. In the first group the penicillin was injected dissolved in distilled water and the effects on the pressure and cell content of the cerebrospinal fluid determined at the end of 6 hours. The second group of experiments was similar to the first except that the animals were allowed to survive 24 hours after the injection of the penicillin. In the third group, the penicillin was dissolved in 0.9 per cent (w/v) sodium chloride solution before injection. The effect of the introduction of 10,000 i.u. penicillin in distilled water was studied in twelve dogs: seven were allowed to survive for 6 hours and five for 24 hours. All animals presented evidence of irritation of the

TABLE I

THE EFFECT ON THE CELL CONTENT AND THE PRESSURE OF THE CEREBROSPINAL FLUID OF 10,000 I.U. PENICILLIN AND OF 0.9% NaCl SOLUTION

Penicillin in distilled water				Penicillin in distilled water				0.9% NaCl in distilled water			
Wt. of dog (kg.)	White cells per cu.mm. 6 hr. after injection	Pressure of C.S.F.		Wt. of dog (kg.)	White cells per cu.mm. 24 hr. after injection	Pressure of C.S.F.		Wt. of dog (kg.)	White cells per cu.mm. 6 hr. after injection	Pressure of C.S.F.	
		Before injection	6 hr. later			Before injection	24 hr. later			Before injection	6 hr. later
7.5	4,320	170	130	9.0	340	130	130	9.5	3,250	140	220
12.0	4,230	200	160	11.5	330	140	120	8.5	1,400	140	170
9.5	2,400	130	120	7.5	440	120	90	6.0	3,400	80	220
5.0	1,770	140	160	9.5	170	80	140	10.5	1,070	110	270
8.0	6,790	150	160	10.0	210	100	120	10.0	2,100	170	160
9.0	6,880	110	230					6.0	2,300	125	250
8.5	Destroyed	130	—					10.5	4,500	100	260
Average 8.5	4,398	147	160	9.5	298	114	120	8.7	2,574	123	220

TABLE II

THE EFFECT ON THE CELL CONTENT AND ON THE PRESSURE OF THE CEREBROSPINAL FLUID OF PENICILLIN IN DOSES LESS THAN 10,000 I.U. AND OF DISTILLED WATER

Wt. of dog (kg.)	i.u. penicillin in distilled water	White cells after 6 hr. per cu.mm. C.S.F.	Pressure of C.S.F.		Wt. of dog (kg.)	i.u. penicillin in 0.9% NaCl solution	White cells after 6 hr. per cu.mm. C.S.F.	Pressure of C.S.F.	
			Before injection	6 hr. later				Before injection	6 hr. later
9.4	7,500	340	140	140	9.4	1,000	6,000	80	230
8.2	7,500	415	180	160	12.0	1,000	7,800	130	260
10.0	7,500	600	130	160	10.5	1,000	7,500	120	200
Average 9.2	—	450	150	153	8.6	1,000	4,700	120	170
11.0	5,000	170	180	180	Average 10.1	—	6,500	112	215
9.0	5,000	280	80	80					
10.0	5,000	140	110	120					
						Distilled water			
Average 10.0	—	197	123	127	10.5	—	0	160	180
8.0	2,500	120	80	100	11.5	—	90	150	160
9.0	2,500	50	80	100					
					8.5	—	0	120	110
Average 8.5	—	85	80	100	7.5	—	80	150	170
8.0	1,000	35	120	140	8.6	—	0	130	110
9.5	1,000	40	170	170					
10.0	1,000	20	90	110	Average 9.3	—	34	142	146
7.5	1,000	35	110	85					
Average 8.7	—	32	122	126					

nervous system after recovery from the anaesthetic. The symptoms most commonly encountered were weakness of the forelegs in walking, slight head retraction, chattering of the jaws with salivation, and attempts to scratch the back of the head with the hind leg. Head shaking was a prominent symptom in some animals. With the exception of one dog which passed into status epilepticus after half an hour, convulsions were not observed. These symptoms generally disappeared after one hour and the animals appeared normal at the end of the 6-hour and 24-hour recovery periods. In four experiments the penicillin was dissolved in sodium chloride solution before injection. There was evidence of severe irritation of the central nervous system in all four experiments on recovery from the anaesthetic, and the animals were destroyed after the onset of general convulsions within half to one hour of the administration of the penicillin. The results of the experiments are summarized in Table I, where, for the purpose of comparison, the effect of the injection of 1.0 c.c. of 0.9 per cent (w/v) sodium chloride solution after 6 hours is also indicated. The limited number of experiments in each group did not seem to warrant a statistical investigation of the results.

The effect of the intracisternal injection of crystalline penicillin in quantities less than 10,000 i.u.

Penicillin dissolved in distilled water was injected into the cisterna magna in the following doses: 7,500 i.u. in 3 dogs ; 5,000 i.u. in 3 dogs ; 2,500 i.u. in 2 dogs ; 1,000 i.u. in 4 dogs ; and 1,000 i.u. in sodium chloride solution was injected in 4 dogs. The duration of the experiments was 6 hours. The effect on the cell count and on the pressure of the cerebrospinal fluid is indicated in Table II. There were symptoms of irritation of the nervous system in all experiments except after the injection of 1,000 i.u. ; after 2,500 i.u. these symptoms were barely perceptible. Although the injection of 1,000 i.u. in sodium chloride solution caused the appearance of a considerable number of polymorphonuclear leucocytes and a rise in the pressure of the cerebrospinal fluid, the animals presented no symptoms of irritation of the nervous system. It is evident that the white cell content and the pressure of the cerebrospinal fluid give no indication of irritant action on nerve tissue.

Discussion

In the above experiments, dogs of an average weight of 8.7 kg. were able to tolerate an intracisternal injection of 1,000 i.u. penicillin in 1.0 c.c. distilled water without displaying symptoms of irritation of the nervous system. Although the pressure of the cerebrospinal fluid remained unchanged after 6 hours, polymorphonuclear leucocytes were invariably present, but their number never exceeded fifty per cu.mm. of cerebrospinal fluid (Table II). These findings contrast markedly with the effects which followed the injection of a similar volume of normal saline solution (Table II). Although symptoms of irritation of the nervous system were not observed after the injection of normal saline solution, the pressure of the cerebrospinal fluid was raised and the polymorphonuclear leucocytic content of the cerebrospinal fluid averaged 2,574 cells per cu.mm. at the end of the experiments.

It will be seen from Table II that 1,000 i.u. penicillin dissolved in normal saline solution provoked the appearance of an even greater number of leucocytes and also a rise in the pressure of the cerebrospinal fluid. The unsuitability of normal saline solution as a solvent for penicillin required for introduction into the subarachnoid space is well illustrated by the experiments in which 10,000 i.u. penicillin were administered. In four experiments in which the penicillin was injected dissolved in normal saline solution, the animals developed general convulsions after half to one hour and were destroyed. On the other hand, convulsions occurred in only one animal out of a series of twelve when the penicillin was dissolved in distilled water, although there was evidence of irritation of the nervous system in every instance.

The irritant action of penicillin on the nervous system seems to be of relatively short duration. Marked evidence of irritation may be present on recovery from the anaesthetic, as was observed after the introduction of 10,000 i.u. in distilled water, yet with the exception of one animal that had to be destroyed owing to the onset of general convulsions the remainder appeared normal one hour later. Similarly the number of polymorphonuclear leucocytes in the cerebrospinal fluid 24 hours after the injection of 10,000 i.u. penicillin was of the same order as that found after the injection of distilled water and considerably less than after the injection of normal saline (Bedford, 1948a). These findings suggest that penicillin may disappear relatively rapidly from the nervous system after intracisternal injection.

It is interesting to note that the injection of normal saline causes the appearance of considerably more leucocytes than the injection of crystalline penicillin in a dose capable of exciting

symptoms of irritation of the nervous system. As already stated, a rise in the pressure of the cerebrospinal fluid followed the injection of normal saline which was never found after the injection of penicillin in distilled water. These findings would seem to indicate that the irritant action of sodium chloride solution is restricted mainly to the covering membranes of the nervous system and possibly to the arachnoid villi. Penicillin, on the other hand, rapidly penetrates the membranes, with the production of relatively little irritation. Its main action is on the nerve cells and their processes rather than on the supporting tissue.

Attempts to discover whether degree of dilution or rate of administration have any influence on the effect produced by a given dose of penicillin have so far yielded indeterminate results. Owing to the small size of the cisterna magna of the dog, it would seem probable that better results would be obtained by making observations on the human subject.

Summary

A study has been made of the effect of the injection of crystalline penicillin into the cisterna magna of the dog. The duration of the experiments was 6 hours except in a few instances, where it was 24 hours. The injection of 1,000 i.u. penicillin in distilled water did not produce symptoms of irritation of the nervous system, the number of polymorphonuclear leucocytes in the cerebrospinal fluid after 6 hours was no greater than after the injection of a similar volume of distilled water, and there was no rise in the pressure of the cerebrospinal fluid. No symptoms of irritation of the nervous system followed the injection of a similar dose of penicillin dissolved in 0.9 per cent (w/v) sodium chloride solution, although the cerebrospinal fluid contained a considerable number of polymorphonuclear leucocytes and its pressure was raised. Doses of penicillin greater than 10,000 i.u. invariably caused convulsions, and this also occurred when 10,000 i.u. were injected dissolved in sodium chloride solution. Convulsions, however, occurred in only one of twelve dogs when 10,000 i.u. were injected dissolved in distilled water, although there was evidence of irritation of the nervous system in every instance. A rise in the pressure of the cerebrospinal fluid never followed the injection of penicillin in distilled water.

The significance of these findings is discussed.

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STUDIES WITH A RADIOACTIVE SPINAL ANAESTHETIC

BY

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The need for information about the fate of a spinal anaesthetic after its introduction into the spinal theca was early recognized (Miller, 1901), but many years elapsed before the problem was seriously studied by a number of workers, including Stout (1929), Küstner and Eissner (1930), Koster *et al.* (1936, 1938, 1939), Bullock and Macdonald (1938), and Shields (1942). In the main these workers agreed that after the intrathecal injection of procaine there was a rapid initial drop in local concentration with a subsequent period of gradual decrement.

Evidently an anaesthetic departs from the subarachnoid space, but what is its route of departure? There are various theoretical possibilities:

(1) Cephalic transit in the spinal cerebrospinal fluid to the cranial subarachnoid space with possible absorption by the cranial arachnoid villi.

(2) Absorption by spinal cord and/or nerve roots with or without a period of "fixation" in these tissues.

(3) Transport *via* the lymphatic drainage of the spinal theca.

(4) Absorption into the venous drainage of the spinal theca either directly or *via* spinal arachnoid villi (Elman, 1923).

(5) A combination of any of the foregoing.

It is proposed to describe a study of the fate of a spinal anaesthetic with special reference to its ultimate distribution among the tissues of the body and the routes by which it leaves the spinal theca.

Method of assay and anaesthetic employed

The method of assay had to satisfy two main criteria: (1) indifference to the nature and amount of the diluting media; (2) a high degree of sensitivity; the former since it was proposed to undertake analyses both in body fluids and various tissues, and the latter since it was not intended to employ disproportionate doses of the anaesthetic. Of chemical methods the diazo-reaction is probably one of the most sensitive for procaine assay: Küstner and Eissner (1930) and Willstaedt (1934) claimed sensitivities to 0.01 and 0.005 mg. respectively for their techniques, while Bullock and Macdonald (1938) asserted that concentrations below 0.02 per cent were beyond their powers of estimation. It was anticipated that the tissue concentration after a normal spinal anaesthetic in the cat would usually be below any of the limits given above, while the gross excess of tissue bespoke laborious separation techniques in which the accumulated error promised to be large even if enough drug were present to render assay possible.

A tracer technique seemed, therefore, to be indicated. Although theoretically any of the atoms of the procaine molecule is open to substitution by its radioactive counterpart, such a procedure was found impracticable when this research commenced because of limited half life, uncertainty of supply, difficulties of assay, or synthesis with the elements normally present. It was therefore decided to introduce some other element into the benzene nucleus without radically changing the pharmacology of the anaesthetic. The halogens were the obvious choice, and preliminary experiments showed that of these Br^{82} was the most satisfactory. Procaine was accordingly brominated by the method of Morel, Leulier, and Denoyel (1929), the necessary hydrobromic acid being prepared as described by Howarth (1948). In this description the author made use of calculated quantities of procaine in the bromination stage, but subsequent experience has shown the reaction to be sufficiently easily controlled to render this unnecessary.

The method was as follows: the concentrated hydrobromic acid having been obtained, 3 or 4 c.c. of 100 vols. H_2O_2 were added to it followed by a knife

point of procaine. The solution in the tube promptly developed a yellow tinge. Further procaine was then added and the coloration disappeared, to return again after a minute or two when more procaine was added. This procedure was repeated until the reaction was completed. In this way the speed of bromination was increased and the necessity for time-consuming estimations eliminated.

The bromine for these experiments was obtained either as KBr from the cyclotrons of Liverpool or Cambridge or as ethylene dibromide from the small or large pile at Harwell. When the small pile was employed 3–6 ampoules each containing 10 c.c. of ethylene dibromide and 5 per cent aniline (Le Roux *et al.*, 1939; Lu and Sugden, 1939) were irradiated, the total activity per ampoule being of the order of 200 millicuries. With the large pile (factor of 10) 1–2 c.c. were irradiated. After this the bromine was extracted as KBr by the Szilard-Chalmers technique (1934).

The dibromoprocaine hydrobromide $C_{13}H_{18}Br_2N_2O_2$, HBr (DBP hydrobromide) thus obtained was converted to DBP hydrochloride either by the method of Morel *et al.* (1929) or latterly by precipitating the base with N/10 NaOH, dissolving the latter in ether, separating and precipitating the hydrochloride with a stream of HCl gas. The entire operation from KBr to DBP hydrochloride occupied about three hours.

Assay was carried out by means of a standard Dynatron X 200 scaling unit and a counter designed to take 10 c.c. of liquid. Body fluids were assayed directly; tissues were broken down in alcoholic lithium hydroxide (Howarth, 1949a) and suitable dilutions of the suspension employed.

Preliminary studies

Three preliminary observations were needed:

(1) Investigation of the spinal anaesthetic properties of DBP.

(2) An assessment of the accuracy of the author's method of radioassay.

(3) Study of the fate of brominated benzene rings in the body.

(1) *DBP as a spinal anaesthetic*

Morel *et al.* (1929) in their description of DBP did not include studies of its applications in spinal anaesthesia.

The anaesthetic properties of DBP were examined as follows: six cats were premedicated with atropine and lightly anaesthetized with ether; each received 10 mg. DBP, HCl in 1 c.c. H_2O at 40° C. by lumbar puncture and the ether administration was then discontinued.

All showed relaxation of the anal sphincter (lower sacral block), loss of knee jerks (lumbar block), but no obliteration of the elbow jerk (lower cervical roots). The hindlimbs were atonic and could be placed in exaggerated postures. On recovering consciousness all the animals appeared paralysed from the lower costal margins downwards; they dragged their hindlimbs. They displayed no interest in violent stimuli administered to their hindlimbs, contrary to the effects of similar stimuli to their forelimbs. In one animal there was evidence of some degree of intercostal paralysis as shown by reduced thoracic and increased diaphragmatic excursions. All the animals recovered completely and walked perfectly within 24 hours. None showed tail droop after this period. It was concluded that DBP was a powerful spinal anaesthetic but with limited usefulness owing to its low solubility. Although only six cats were examined under these controlled experimental conditions, the author has administered DBP to some 200 cats and six monkeys without producing any persistent ill effects except in four cases, and in at least two of these a persistent paraplegia was due to haematomyelia after lumbar puncture.

(2) *Accuracy of radioassay*

In order to investigate this an anaesthetized cat received 0.5 c.c. of a DBP, HCl solution by lumbar puncture. Six samples of cerebrospinal fluid were collected and each assayed by the author and by Dr. K. Bullock, who kindly undertook the chemical estimations. The basis of the latter was diazotization and coupling followed by photocolorimetry. Standards prepared gravimetrically were also separately assayed. The results showed a satisfactory agreement (Table I).

TABLE I

CONCENTRATION OF DIBROMOPROCAINE HCL IN SUCCESSIVE SAMPLES OF CEREBROSPINAL FLUID (G./100 C.C.)

Sample Number	Estimation Diazo Dr. Bullock	Estimation Radioassay Author
1	0.043	0.050
2	0.037	0.041
3	0.022	0.024
4	0.011	0.012
5	0.005	0.0041
Vol. of C.S.F. assayed ...	0.1 c.c.	0.02 c.c.

Estimation of a standard solution (g./100 c.c.)

Gravimetric	0.015
Diazo assay	0.018
Radioassay	0.016

In later experiments closer agreement has been reached.

(3) *Fate of DBP*

On account of its great stability it is unlikely that DBP suffers removal of its nuclear halogen atoms during its sojourn in the body (Fromherz, 1928 ; Williams, 1947). Nevertheless, from the point of view of laboratory manipulation, stability of a halogen attached to a benzene ring within an organic compound is influenced by the arrangement of the various groupings about such a ring. Thus it was expedient to investigate the drug from this point of view, since the removal of a large proportion of tagged atoms would severely reduce their value as a tracer for this molecule.

The method was as follows:

An anaesthetized cat received by the preaxial vein of the forelimb 0.1 g. DBP in 20 c.c. water, injection being made slowly so as to avoid cardiac or respiratory depression. After two hours the animal was sacrificed, the abdomen opened, and the urine aspirated from the bladder ; 1 c.c. of the urine was placed in each of five small tubes. To each of these were added 2 c.c. of a NaBr solution to serve as a carrier, and to four of them 0.5 c.c. of a saturated solution of $AgNO_3$. The precipitate of AgBr was filtered off and washed thoroughly, the filtrate being made up to 10 c.c. and subjected to radioassay. The figures obtained were compared with those derived from the tube containing no $AgNO_3$. The results (Table II) showed that most of the bromine atoms in the urine did not exist as ionizable bromine, but as bromine in organic combination. The last two lines of the Table indicate that the anaesthetic itself, prepared by the methods described, did not contain ionizable bromine.

It has been shown recently that even after five days the urinary bromine exists chiefly as bromine in organic form.

TABLE II

This table shows that the radioactivity of urine after intravenous dibromoprocaine hydrochloride was not reduced by treatment with sodium bromide (carrier) and silver nitrate followed by filtration. The radioactive bromine atoms have not been precipitated by the silver nitrate; therefore the bromine is organically bound. The last two lines show that there is no ionizable bromine in the anaesthetic itself.

Exp. No.	Contents of tube (c.c.)				Standardized counts per min.
	Urine	Silver nitrate	Sodium bromide	DBP HCl soln.	
1	1	0	2	0	684
2	1	0.5	2	0	642
3	1	0.5	2	0	651
4	1	0.5	2	0	656
5	1	0.5	2	0	684
6	0	0.5	2	1	160
7	0	0	2	1	165
	Statistical error ± 4% S.D.				

Note on interpretation of results

The concentration of DBP hydrochloride in an aqueous solution was estimated by comparing its counting rate with that of a standard of known concentration. In the tissues, however, procaine and probably DBP is broken down into a number of products (Burgen *et al.*, 1948 ; Goldberg *et al.*, 1943). In the present work the results of comparison with standard solutions of DBP are expressed as μg. of the latter per g. (or c.c.) of tissue. This should be understood as that amount of DBP, HCl which would be present if all the benzene nuclei were incorporated in molecules of DBP.

Distribution in Body Fluids

In this series of experiments it was decided to investigate the levels of DBP obtaining in the C.S.F., blood, and urine after intrathecal injection.

Method

Cats anaesthetized with pentobarbitone received various doses of DBP, HCl by lumbar puncture. The needle was left *in situ* with stilette in position, but at fixed time intervals the stilette was withdrawn, and, after permitting a few drops of C.S.F. to escape, the inside of the butt of the needle was cleaned with a spill of filter-paper and 0.02 c.c. of C.S.F. was collected in a blood pipette. Blood was taken at similar time intervals from a cannula inserted into the femoral or carotid artery. Urine was obtained by suprapubic cannulation of the urethra, the cannula being tied in position and led by way of a narrow rubber tube into a small collecting phial. The phial was changed at 15-min. intervals, the last of the urine being expressed by gentle pressure over the bladder just before the change of receiver. Further specimens of C.S.F. were obtained from the cisterna magna by means of a hypodermic needle passed through the atlanto-occipital membrane, the volume removed at each sampling being 0.1 c.c. The various specimens obtained were subjected to radioassay.

Results

The graph (Fig. 1) shows the results obtained from one experiment. The main features are the rapid initial decline of the drug concentration in the C.S.F., followed by a less steep phase, the rapid rise of urine concentration followed by a slower fall, and the low steady blood level of drug. The concentration within the cisterna was usually so low as to render accurate assay valueless.

Five animals were studied in detail and the curves for urine, C.S.F., and blood found to be similar in form for each animal though there was some individual variation ; thus though the decline of the concentration of C.S.F. was always rapid, the actual rate of fall varied. There appeared to be

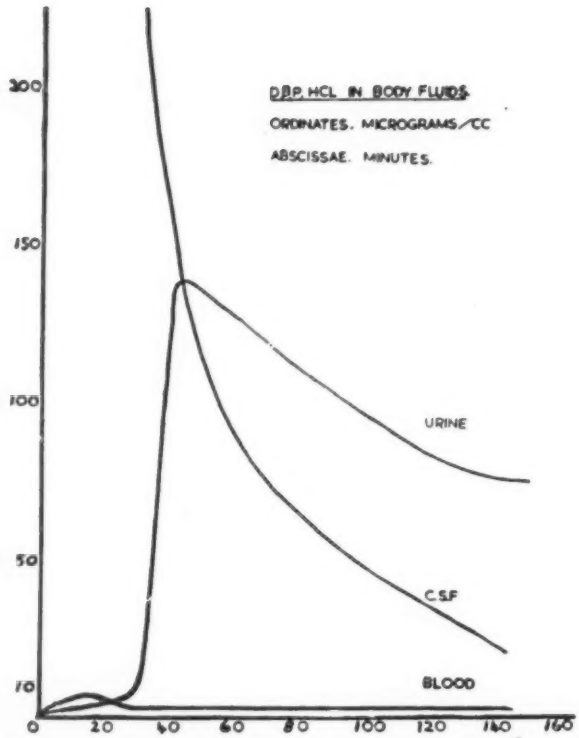

FIG. 1.—Dibromoprocaine levels in body fluids after spinal subarachnoid injection.

no simple relationship between rate of decrement in concentration, dose of drug, and weight of animal. The " bump " on the blood level seemed to correspond to the period of maximum decline of the C.S.F. concentration. The form of the urine curves presented no striking differences except that the peak level varied both as regards time and maximum height.

TISSUE DISTRIBUTION

The observation that DBP rapidly leaves the theca, appears in the urine in high concentration, and maintains a persistently low blood level made it of interest to study the distribution of the drug among the body tissues. A preliminary note of this study has been published (Howarth, 1949b).

Methods

After subarachnoid injection

The cats were anaesthetized with pentobarbitone, the carotid artery cannulated, and a spring clamp placed upon the vessel proximally. The bladder was catheterized and a dose of DBP administered by lumbar puncture, the needle and stilette being left *in situ*. The presence of spinal anaesthesia was confirmed and the animals left for a specific period. Fifteen minutes before autopsy the bladder was emptied and the catheter clipped so as to collect within the bladder a 15-minute specimen of urine. Preparation was now made for autopsy, and a sample of blood (1–2 c.c.) was taken in a small tube and shaken with a few crystals of sodium citrate in order to prevent clotting. The clamp was removed from the catheter and the urine collected. The stilette was withdrawn from the lumbar puncture needle and, after allowing two or three drops of C.S.F. to escape, 0.02 c.c. of C.S.F. was collected in a blood pipette and discharged with washing into about 4 c.c. of water.

Autopsy

Autopsy was performed in the usual manner, special efforts being made to prevent loss of blood.

The spinal cord was removed complete with membranes whilst the animal was still alive, so as to reduce post-mortem absorption of DBP remaining in the cerebospinal fluid. The dura mater was stripped from the spinal cord and the roots of the cauda equina divided close to the cord and retained for assay. One centimetre of cord was removed from the lumbar region at the site of introduction of the DBP. Neither roots nor cord were cleaned, and consequently some of the administered anaesthetic adhered to their surfaces.

Removal of samples

In removal of specimens from an organ adherent blood was removed from its surface and a small section excised, free blood being gently swabbed from the cut surface. An endeavour was made to remove the same portion of each organ in each experiment. A list of samples is appended with relevant notes.

Cord and roots (see above).

Sciatic nerve in its entirety.

Medulla oblongata from the level of the atlas to the lower border of the pons.

Spleen about half an inch from its left-hand extremity.

Liver. A portion from the middle of the anterior surface of the right lobe.

Bile aspirated from gall-bladder after liver specimen had been removed.

Kidney. This was incised along the convexity and the organ decapsulated by pressing it through the incision. The kidney was divested of cortex with a razor blade starting at the attachment of the ureteric pelvis which marks the cortico-medullary junction. Cortex and medulla were assayed separately.

Muscle. The bellies of the left hamstrings.

Lung. Lower quarter of the right lung.

Gut contents. These consisted of the contents of the third part of the duodenum and were extracted by massage of the isolated length of gut held over a watch glass.

Gut. A portion of the second part of the duodenum.

Thyroid and testis (or ovary) in their entirety.

Skin. A portion from the scalp shaved prior to autopsy.

TABLE III

TISSUE/BLOOD RATIOS (*Ct/Ca*) AFTER INTRATHECAL INJECTION OF DIBROMOPROCAINE HYDROCHLORIDE

Weight of cat (kg.) ...	3.6	2	3.5	2.1	3	2.5	2.25
Dose (g.)	0.007	0.007	0.025	0.007	0.008	0.03	0.005
Time after injection (hr.)	1	1.23	2.5	3	21	39	20
Blood*	3.36	5.41	3.34	2.73	1.31	2.86	0.78
Spleen	0.26	0.25	0.24	0.30	0.50	0.22	2.51
Medulla	0.091	0.11	0.074	0.13	0.30	0.12	0.25
Sciatic nerve	0.27	0.37	0.2	0.48	0.84	0.31	0.63
Muscle	0.11	0.1	0.049	0.067	0.13	0.074	0.19
Lung	0.73	0.52	0.47	0.67	0.80	0.44	0.50
Skin	0.5	0.32	0.31	0.63	0.93	0.58	1.29
Thyroid	0.49	0.27	0.19	0.4	0.80	—	—
Testis	0.53	1.5	0.28	0.54	0.6	0.30	—
Gut	0.34	3.1	0.48	0.56	0.6	0.29	0.59
Kidney cortex	8.43	9.3	6.2	7.2	1.3	0.36	3.40
Kidney medulla ...	6.4	4.3	2.6	2.7	1.5	0.88	1.80
Liver	2.3	2.8	1.7	1.4	1.1	0.34	1.35
Bile	6.73	26.6	7.2	8.6	9.8	0.67	5.7
Urine	83	27	24	133	4	0.8	15.7
Cord*	38.3	26	26	8.4	0.64	0.6	0.55
Roots*	163	94	76	76	1.6	0.8	0.87
C.S.F.*	103	37	19.7	22	1.3	1.7	—
Duodenal contents* ...	6.76	92	7.2	18.3	None	3.6	—
Error	3–4%						

* Expressed as μg./g.

Estimation

Specimens were placed on watch glasses on a tray over which a wet cloth was suspended in order to reduce evaporation. The specimens were weighed within a few minutes of removal from the body, the smaller organs—i.e., thyroid, nerve roots, and ovary—being weighed immediately. Assay was undertaken as described above.

Results

Table III shows the results obtained from seven animals. The drug concentrations found in C.S.F. cord, roots, and duodenal contents were not directly dependent upon blood concentration, and these were expressed as μg./c.c. or μg./g. of tissue. The rest of the tissue concentrations were expressed as the ratio between mass of drug per gramme of tissue and mass of drug per c.c. of blood (*Ct/Ca* ratio).

The blood contained in the vascular bed of the organ under consideration could not be satisfactorily removed. Thus each assay included an unknown modicum of drug in the blood of its vascular bed. This capillary volume could not be expected to remain constant in different animals. Furthermore, the doses of drug and the time intervals before autopsy were also varied, and hence consistent results were not anticipated. This difficulty has been mentioned by Wallace and Brodie (1937) in their estimations of tissue iodides and thiocyanates.

Of all the tissues examined, apart from those in direct contact with the drug in the C.S.F., only liver and kidney showed a tissue concentration above that obtaining in the blood (tissue/blood ratio). Of these the kidney values were much the higher, and in the majority of the assays the cortex showed a higher concentration than the medulla. The concentration in the duodenal wall appeared puzzling and erratic until it was found that the bile contained an appreciable amount of DBP. It is interesting to note that both blood and C.S.F. contained detectable amounts of DBP 39 hours after its injection.

The concentrations found in the cord and roots show special features. In all but one experiment, the drug concentration was lower in the cord than in the C.S.F., but the roots always contained a higher concentration of drug than the cord. It appeared that the roots and not the cord were capable of concentrating the drug from the C.S.F. in which they were bathed.

The medulla oblongata showed a very constant tissue/blood ratio, and such constancy would not be expected had the drug ascended within the spinal subarachnoid space to any significant extent. The tissue distribution after intravenous

TABLE IV

TISSUE/BLOOD RATIOS (*Ct/Ca*) AFTER INTRAVENOUS ADMINISTRATION OF DIBROMOPROCAINE HYDROCHLORIDE

	Values for *Ct/Ca* after intravenous injection					
Weight of cat (kg.)	2.5	3.75	2.5	2.6	2.5	3
Dose (g.)	0.036	0.17	0.092	0.05	0.078	0.12
Time after injection (hr.)	1.20	70	4	2	4	2
Spleen (0.25)	0.27	0.49	0.28	0.2		
Medulla (0.11)	0.14	0.19	0.14	0.12	0.18	0.1
Sciatic nerve (0.33)	0.4	0.5	0.33	0.38		
Muscle (0.08)	0.14	0.11	0.11	0.09		
Lung (0.57)	0.81	0.75	0.71	0.4	0.6	0.55
Skin (0.47)	0.53	0.87	0.30		0.57	
Thyroid (0.34)	0.34		0.53	0.28	0.5	
Testis (0.41)	0.78	0.65	0.71	0.46		
Gut	0.93	0.31	0.08	0.55		
Kidney	5.75	1.23	1.71	2.4		
Liver	2.6	0.38	0.32	1.23	1.06	1.46
Bile	None	0.66	25.6	6.02		
Urine	39	0.27	30.2	30.6		
Cord	0.14	0.17	0.05	0.045	0.12	0.06
Roots	0.27	0.44	0.24	0.22	0.41	0.26
Blood (mean level 3.54 μg./g.) ...	22.1	4.52	33	43.9	11.4	38.8

Figures in parentheses are mean *Ct/Ca* ratios after intrathecal injection.

injection was also studied in order to ascertain whether any difference existed between root and cord concentrations, and to study the effects on tissue/blood ratios of higher blood levels.

The drug was injected into the preaxial vein of a forelimb and assays of organs made at various intervals. The results are tabulated (Table IV). It will be noted that four animals were studied in detail, but that estimations were made of tissue/blood ratios for cord and roots in six animals. For convenience of reference the mean tissue/blood ratios for the intrathecal analyses are included in the left-hand column. In this series of means, the 21-hour-period results are not included; means have not been provided for gut, bile, liver, kidney, or urine, since these results were variable, depending largely upon the state of the animal and the time interval between administration and sampling. In a similar way no means are tabulated for cord and roots since these values are mainly dependent on the concentration of drug in the theca.

The results show that even after intravenous injection the roots reveal a higher concentration of drug than the cord. On the whole the tissue/blood ratios after intravenous injection show a slight general increase, but in view of the relatively enormous blood levels it is doubtful if this increase has any real significance.

The concentration of the drug in the contents of the descending colon was assayed and also the concentration obtaining in the bone marrow of the femur. The values were of the order 2 μg./g. and 0.23–0.34 (tissue/blood ratio) respectively.

Only two observations were made and no assays were undertaken of the entire mass of faeces; the results merely indicated that some of the drug was excreted *via* the gut.

The concentration of drug in the cisterna magna was usually insignificant except in one case where the concentration rose to 1.9 μg./c.c. after 1½ hours. It was of interest, since assays of the cervical cord were planned, to discover the origin of this drug in the cisterna; some had undoubtedly risen from the lumbar regions, but it was possible that some had been secreted by the choroid plexuses.

DBP and the blood/C.S.F. barrier

DBP was administered by the preaxial vein of the forelimb, and specimens of C.S.F. were taken at specific intervals from the cisterna magna. Blood samples were taken at similar periods from the right femoral vein. A graph of such an experiment (Fig. 2) shows that DBP circulating in the blood in sufficient concentration was able to pass the choroid plexus.

Since it had been ascertained that DBP passes rapidly into the blood stream and its distribution

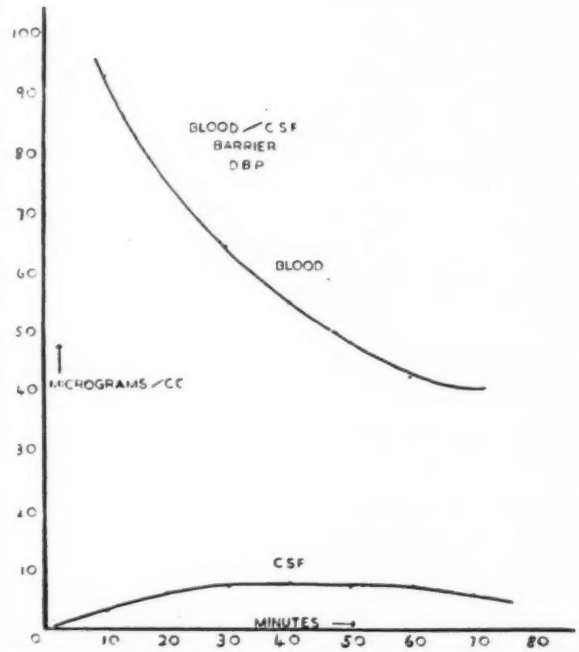

FIG. 2.—Dibromoprocaine appears in low concentration in the cerebrospinal fluid after intravenous injection of 132,400 μg. of DBP, HCl. (Cat 2.5 kg.)

among a number of organs had been studied, it became of interest to investigate the route by which it left the spinal theca.

The possibility of lymphatic drainage will first be considered.

Lymphatic drainage of the subarachnoid space

Since the lymphatics draining the region of the lumbar theca ultimately enter the thoracic duct, it was decided to cannulate this duct above the diaphragm. This was effected in the following manner: Under nembutal anaesthesia the lower part of the thoracic aorta was exposed by the appropriate rib resection, haemostasis being obtained by previous ligation of the ribs in front and behind the proposed resection, and afterwards by diathermy. When the thorax was opened the animal was maintained with artificial respiration, and the lungs were packed away from the aorta. The exposure was extended caudally into the costophrenic angle, and by depressing the cupola of the diaphragm a view of the aortic orifice was obtained. The parietal pleura was dissected away from the right side of the aorta until the thoracic duct appeared like a white thread on the pink aortic wall. The duct was ligated and operations discontinued for 30 minutes.

On recommencing the operation, the distended cisterna chyli could be seen at the aortic orifice. The pleura was removed from the cisterna chyli and a loose ligature cast around the upper part of the latter. A large-bore, curved, and blunted intramuscular needle was used as a cannula, and this was driven by steady pressure into the cephalic end of the cistern and tied in position. A male connector bearing a short length of cycle valve tubing was fixed into the cannula and the wound closed so as to leave an inch of tubing protruding through the chest wall. The chyle flowed freely at first, but the cannula frequently became obstructed. It was then necessary to empty the cistern by gentle aspiration with a syringe. Fig. 3 shows the exposure and cannulation of the thoracic duct.

Introduction of DBP

The muscles of the back are drained in part by the lymphatic channels which enter the thoracic duct (Gray, 1946), and introduction by lumbar puncture was thus unsuitable as a routine procedure since leakage into the muscles of the back was possible; in order to avoid this the theca was exposed where it passes over the sacral promontory and a narrow-bore cannula introduced into the subarachnoid space and tied in position.

Samples of chyle and C.S.F. were taken at frequent intervals after introduction of DBP, HCl. Since the maximal fall of C.S.F. concentration occurred over the first 30 minutes (Fig. 1) it was decided to limit the observations to this period.

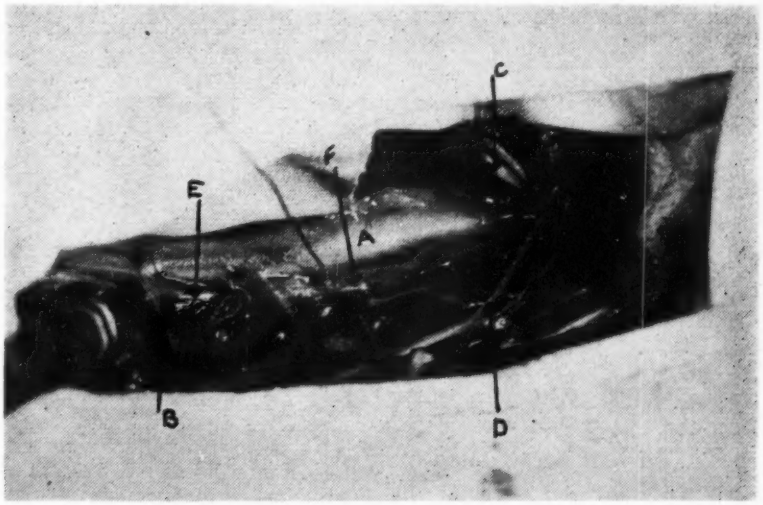

FIG. 3.—Supra-diaphragmatic cannulation of thoracic duct. The chest has been opened: A, aorta; B, intercostal artery; C, crus of diaphragm; D, splanchnic nerves; E, curved cannula and rubber connector; F, tip of cannula in thoracic duct.

Results

Fig. 4 shows that the C.S.F. concentration fell rapidly notwithstanding the new method of introduction and the extensive surgery. All six animals thus investigated showed this. The quantity of

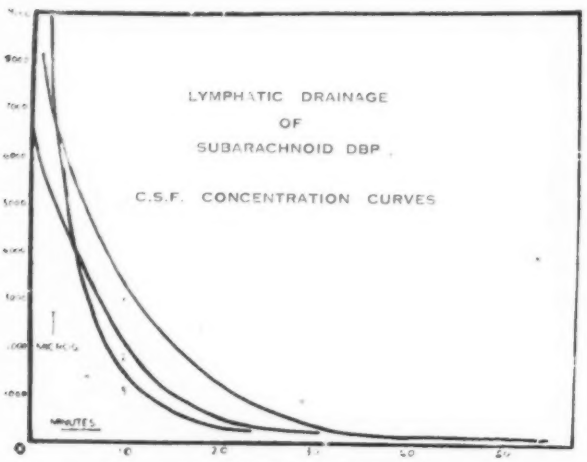

FIG. 4.—The rate of fall of the concentration of dibromoprocaine in the cerebrospinal fluid is rapid despite cannulation of the thoracic duct. The concentration of drug in the duct is too small to show on this scale.

drug excreted by the thoracic duct was in all experiments too low to be represented on the graph. Table V gives the figures for the experiment relating to curve 2 on Fig. 4. It is evident that the total excretion by the thoracic duct is not a considerable factor in the reduction of drug concentration in C.S.F.

In a comparable animal receiving the same dose but administered by lumbar puncture, the total drug excretion in the chyle attained 24.3 μg. in 27 minutes, during which time the C.S.F. concentration fell from 8,620 to 245 μg./c.c.

TABLE V

CAT ♂2.4 KG. DOSE BY SACRAL CANNULA: 12,000 MICROGRAMMES

Time after injection (min.)	DBP in C.S.F. (μg. per c.c.)	Chyle	
		Volume (c.c.)	DBP (μg.)
1	7,800	0.40	0.37
4	5,500	0.30	0.27
8	4,300	0.32	0.26
12	2,900	0.50	0.44
16	1,700	0.40	0.32
21	1,100	0.35	0.63
33	260	0.50	1.14
55	160	0.25	0.31

Total volume of chyle = 3.02 c.c.
Total excretion = 4.24 μg.
= 0.035% of total dose

Passage of DBP into the spinal cord

The possibility of absorption of drug by the intrathecal neural elements was next considered. Previously (Table III) it has been shown that the roots appear to concentrate the drug from the C.S.F. but that the cord does not. There is no information about the concentration within the cord substance itself, and the values obtained include an indeterminate quantity of anaesthetic clinging to the surface. The technique first employed was that of radio-autography.

Method

It is a *sine qua non* in radio-autography that the position of the active substance in the specimen must not change as a result of the application of histological reagents, but though the final form of DBP in the nervous tissues was unknown the difficulties inherent in the solubility of DBP, HCl itself were fully realized. A rapid fixation of the drug in the tissues was necessary to ensure that diffusion would not occur after death.

In an anaesthetized cat laminectomy was performed over the lumbar enlargement. The dura was held apart by small haemostats. The C.S.F. was allowed to escape and the knee jerks elicited. A pledget of wool soaked in 0.5 per cent (w/v) DBP, HCl in water or Dale's Ringer at body temperature was placed on the lumbar enlargement, absent knee jerks indicating the onset of anaesthesia. The pledget was replaced every five minutes until five changes had been made. Five minutes after the last application the spinal cord was clamped above and below the site of medication and the intervening length of cord with its attached roots removed *en masse* from the vertebral canal. The cord was severed just beyond the site previously occupied by the wool, and using the upper clamp as a handle the remainder of the cord was plunged forthwith into a Dewar flask containing liquid air. The cord was frozen solid and the anaesthetized portion isolated with an ampoule saw, placed upon the stage of a freezing microtome, and sections of 30 μ were cut. The sections were laid in the centre of small cells constructed upon coverslips and flattened with a spatula. The coverslips were placed immediately on a metal plate kept "cold" by liquid air. The coverslips were then transferred to small squares of Ilford no-screen *x*-ray film, resting upon small circles of thicker glass. Each cell was placed in a typewriter ribbon box and stored in the refrigerator for a week. A specimen was inspected from time to time to ascertain that it was still frozen, to develop a film, and to inspect the autograph produced at various time intervals. Subsequently all were developed and the sections returned to the refrigerator during the

development period when the coverslips bearing the sections were superimposed on the negative and the area of darkening in the latter ascribed to the relevant parts of the cord.

Figs. 5A, B, and C are autographs obtained by this technique. They exhibit a zone of radioactive anaesthetic surrounding and outlining the periphery of the cord. Within this zone are the nerve roots cut in transverse section, and to demonstrate them a number of sections were rinsed in water before placing them on the coverslips. Figs. 5D and E show the results of this manœuvre, the roots containing a higher concentration than the cord and revealing themselves as intense white spots.

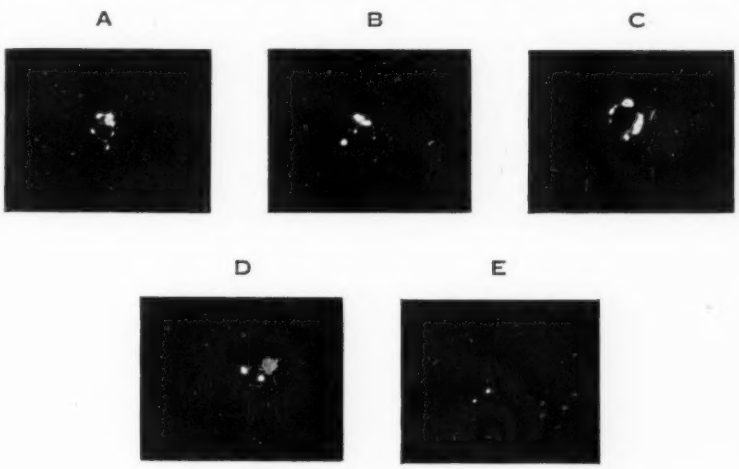

FIG. 5.—Radioactive anaesthetic around a number of cord sections. In D and E most of the anaesthetic has been washed away, but that in the roots remains. These autographs were made by a crude technique before the advances of Bélanger and Leblond (1946) and Pelc (1947). For details see text.

Results of these autographic studies should be interpreted with caution since the edge of the cord could not be related to the edge of the blackened area exactly, and the apparent absence of drug in the centre of the cord may simply indicate a concentration inadequate to affect the film (Hamilton, 1942).

Direct estimation of penetration

It was desirable to determine by some more sensitive method whether any drug had penetrated into the depths of the cord.

Method

In a number of anaesthetized cats, cannulae were inserted into the right common carotid artery and the sacral subarachnoid space. A dose of DBP was introduced by the sacral cannula. A hypodermic needle was passed into the cisterna magna. After a specific interval a specimen of blood was taken from the carotid artery and samples of C.S.F. from both the cisterna and the lumbar sac. After laminectomy the cord and membranes were removed from the upper cervical to the lower sacral region, bleeding being controlled by diathermy and haemostats. The cervical end of the cord was held in artery forceps and the entire cord lowered slowly into a Dewar flask containing liquid air. The frozen cervical and lumbar enlargements were isolated. Specimens were now removed from the centre of the lumbar enlargement. The cervical portion was returned to the liquid air to await attention. The lumbar enlargement, wrapped in gauze and lint, was permitted to thaw until it reached a consistency suitable for cutting. The wrapped piece of cord was held in the hand and the upper half-inch of dura was incised longitudinally on the ventral and dorsal aspects, the two flaps so formed being reflected over the rest of the cord by retraction with toothed forceps. The underlying pia was heavily contaminated with radioactive DBP, and it was essential to avoid contact between this and the tissue.

The pia and periphery of the cord were removed by making thin longitudinal tangential slices all round in such a way that the ends of the slices overlapped. The knife was washed and dried before each cut and the cord returned to the liquid air from time to time in order to maintain its frozen state. In this way a central core of relatively uncontaminated cord was obtained which was severed near the projecting uncut cord.

A chrome steel cylinder having at one end a razor-sharp cutting edge had been prepared and was placed with its cutting edge against the uncontaminated cord. By gentle screwing movements the cylinder was driven through. When a suitable distance had been traversed the cord was incised around the metal cylinder just proximal to its cutting edge. The rest of the cord was gently pulled away from the severed collar until a portion of the cord centre cut by the metal cylinder became visible. This was then cut through. The central part of the cord contained within the cylinder was now driven out a few millimetres by pressure from a metal rod. The end of the protruding cord was cut off and the rest driven out entirely, the other end of the cord also being removed. The remaining rim of cord tissue was inspected to make sure that the cutter had not penetrated the contaminated pial covering; in order to establish this the entire rim of cord tissue was placed in formalin, sectioned serially, and examined microscopically (Fig. 6). Alternatively the

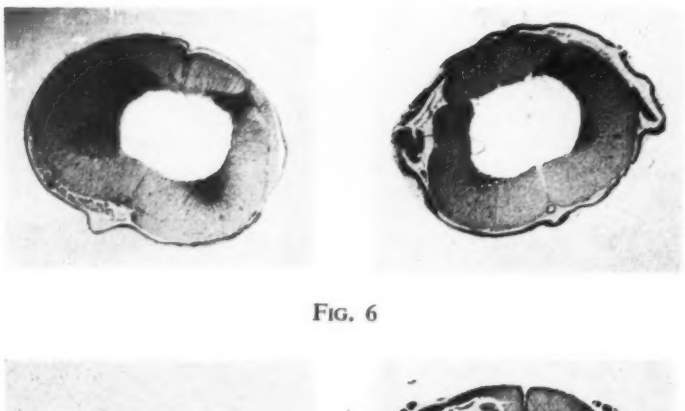

FIG. 6

FIG. 7

FIGS. 6 AND 7.—Sections of the remaining cord tissue after removal of the deeper portions (6) with a cylindrical cutter, (7) with an iris knife. In all sections the peripheral portions are intact.

rim was bisected transversely and the upper portion assayed and the lower sectioned as above.

The central rim of the cord was then weighed, broken down in lithium hydroxide, and radioassayed. The cervical cord was assayed in a similar manner.

In some of the sections it was found that the tip of the anterior median fissure was included in the removed cord centre. It is improbable that DBP entered the fissure in any significant amount since it was not outlined in the autographs; nevertheless in order to eliminate this, after the previously described precautions had been taken, a small portion of each lateral white column of the spinal cord was removed with an iris knife and assayed, sections being made of the peripheral rim as before. Examples of such sections are shown in Fig. 7. It will be seen that the anterior median fissure has not been encroached upon. The results of assay were comparable with those of former experiments.

Results

The results (Table VI) show that the blood concentration of drug is high enough to be easily assayed in five minutes after subarachnoid injection. There is a possibility, therefore, that the tissue concentrations obtained are due to the blood in their capillary beds rather than to DBP which has directly penetrated the cord. Studies have already been made of the tissue/blood ratios in respect of the cord after intravenous administration of the drug. The observed results of the cord centre concentration are similarly expressed, and it will be seen from Table VI that the ratios calculated for this series of experiments are far higher for both the cervical and the lumbar cord centres than the corresponding ratios after intravenous injection. DBP has entered the cord by some route other than the blood stream, presumably through the pial membrane in the case of the lumbar cord.

The results recorded for the cervical cord are of special interest, for here it can be seen that the

TABLE VI

DISTRIBUTION OF DIBROMOPROCAINE HYDROCHLORIDE WITHIN THE INTRATHECAL NEURAL ELEMENTS

All values expressed as μg./g. or μg./c.c. unless otherwise stated*

Time (min.)	5	10	20	20	25	25
Dose (g.)	0.012	0.0033	0.0033	0.0033	0.0033	0.012
Roots	1,000	320	246	355	63.7	400
C.S.F. lumbar	3,900	1,340	106	190	273	203
Cord periphery lumbar	520	138	66	138	30.9	300
Cord centre lumbar	120	5.3	2.6	5.8	18	49
Cord periphery cervical	17	0.5	0.42	0.7	6.5	2.3
Cord centre cervical	7	0.7	2	0.8	0.5	1.9
C.S.F. cervical	3	±00*	±00*	±00*	5.4	0.9
Blood level	2.9	0.646	0.692	0.78	0.877	2.7
Cord centre lumbar *Ct/Ca*	41.4	8.21	3.76	7.44	20.5	18.1
Cord centre cervical *Ct/Ca*	2.41	1.08	2.39	1.03	0.57	0.705

* ±00 = Too small to estimate. *Ct/Ca* cord ascertained in previous experiment (Tables III and IV) always less than 0.2.

concentration in the cord periphery is higher than that in the C.S.F. which bathes it. There are three possible explanations for this. Either the drug has ascended from the lumbar regions *via* the pial plexus of veins, or by the neural elements within the cord, or the cervical cord has specifically absorbed and concentrated the drug locally from the C.S.F. Specific concentration is unlikely in that it is not a feature of the behaviour of the cord in contradistinction to the roots (Table III). Spread within the cord is likely, since Brierley and Field (1948) have shown that phosphorus injected into the sciatic nerve can be found in the upper reaches of the spinal cord. This passage upwards in the deeper parts of the cord is probably not within the deep veins, since these drain transversely into a superficial longitudinal venous plexus (Mettler, 1942) and do not travel the length of the cord within its substance. It is the results obtained for the cervical cord centres that make some degree of cord penetration by a spinal anaesthetic reasonably certain.

It will be seen that tissue/blood ratios for cord centre and cord periphery have been compared with tissue/blood ratios established for the entire cord. It is possible that tissue/blood ratios for cord centre and cord periphery differ. In order to elucidate this a monkey received an intravenous dose of DBP and the ratios were ascertained for both the central and peripheral parts of the spinal cord. They proved to be 0.21 and 0.23 respectively.

It must be emphasized that it is not possible to compare results obtained from different animals since cord diameters vary and the cutter did not. Consequently a central core removed from a cord of small diameter will encroach further upon the periphery than a similar core removed from a cord of large diameter, and it is in these peripheral portions that a higher concentration of anaesthetic is to be expected.

Note on fixation

Neural fixation has been stated to be responsible for the decrement of drug concentration in the C.S.F. ; further, it has been claimed (Stout, 1929) that such fixation prevents ascent of the drug to the vital centres. This is untenable. It has never been suggested that sodium, phosphorus, or bromine suffer such fixation ; none the less, compounds of these elements do not appear within the cephalic reaches of the spinal subarachnoid space in any significant amount after intrathecal injection. Further, the rapid appearance of these substances in the venous system clearly points to a more likely mechanism (Howarth and Cooper, 1949).

Of the neural elements within the theca only the spinal roots show regularly a capacity to concentrate a spinal anaesthetic to a level above that in the C.S.F. ; to this phenomenon alone is the term " fixation " applied with precision.

" Fixation " implies retention ; though this may occur, the total quantity of drug involved must be small since there is no appreciable arrest of the drug within the theca, the anaesthetic appearing almost at once in the venous drainage of the part. In the decrement curves there is no striking difference between those of DBP, sodium, phosphorus, and bromine, and there is no reason to believe that any of the latter three substances is retained within the theca (Howarth and Cooper, 1949). Even in the roots the total amount of DBP retained must be small.

The problem of neural block by the anaesthetic within the cord

In the last section it was shown that DBP could penetrate the spinal cord, and it was of interest to discover whether the concentration there was adequate to block the pathways within the cord. The respiratory pathway was selected for study. According to Starling's textbook (1936) this pathway conveying impulses from the respiratory centres to the cells of origin of the intercostal nerves is situated in the deeper portions of the lateral columns of the cord. Pitts (1940) localized this pathway in the anterior and antero-lateral columns of the cord. Accordingly it was decided to apply an anaesthetic to the cord in an attempt to block this pathway.

Method

In all 45 cats and four monkeys were examined. Laminectomy was performed on the anaesthetized animal extending from the 6th cervical to the 2nd or 3rd thoracic vertebra. Bleeding was controlled and the dura incised along the midline between the caudal and cephalic extremities of the wound. The dural flaps were removed until only a small band of the membrane remained on the ventral aspect of the cord. A swab moistened with warm Dale's Ringer was placed in the wound and a cannula was inserted into the femoral artery and connected to a blood-pressure apparatus. Tambours were placed on the middle of the thoracic wall and upon the antero-lateral abdominal parietes so that thoracic and abdominal respirations could be recorded simultaneously.

The animal was placed upon a board with its caudal end elevated through an angle of 60°, the animal's

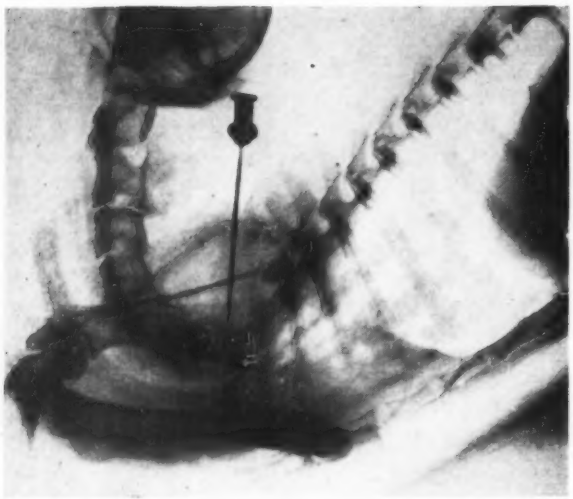

FIG. 8.—Skeletal relationships before application of anaesthetic to cervical cord. Needle indicates the middle of a three-segment laminectomy.

head raised by means of a clamp fixed to its lower jaw, and the extension of the neck continued to the limit of its free movement.

Thus the lower cervical part of the cord was placed in the depths of the wound at the lowest part of the U (Fig. 8). In this way spread of the anaesthetic placed in the base of the U was reduced, sparing the phrenic roots proximally and the intercostal nerves distally.

The smoked drum was set in motion and after a short interval a pledget of wool bearing a warm solution of anaesthetic was placed on the cord and replaced by a further pledget in five minutes. This was repeated throughout the experiment, care being taken to ensure that the anaesthetic had access to the entire periphery of the cord.

Results

The results were consistent and no interruption of the tract was observed after the application of DBP, procaine, cinchocaine, amethocaine, or cocaine itself. Fig. 9 shows the end of such a trace after application of amethocaine followed by percaine.

Before a satisfactory interpretation could be attempted it was essential to discover if a spinal anaesthetic could produce block of the pathways in the cord even if it were placed in contact with them. Thus a small volume of dilute procaine was injected directly into the cord with a fine needle, after first making a small incision in the pia. Under these conditions block of the respiratory pathway was produced with 0.2 c.c. of a 0.5 per cent (w/v) procaine solution (Fig. 10). Neither saline nor water produced such an effect. In Fig. 11 the phrenic roots were paralysed after cinchocaine at T1 by lowering the head, and there was a compensatory increase in thoracic respiration. Later the respiratory centre was paralysed and all respiration ceased. Lowering the thorax did not regularly produce intercostal paralysis, and it was necessary to inject the drug under the free edge of the dura into the thoracic subarachnoid space.

Venous drainage of the spinal subarachnoid space

Various routes whereby a spinal anaesthetic may leave the subarachnoid space have been considered, but none has shown itself in an unequivocal manner to be pre-eminent. Since the appearance of the drug in the blood stream is almost immediate (Howarth and Cooper, 1949) it seemed that a direct venous drainage was the most likely route of departure for a substance administered intrathecally. Further studies have in a large measure confirmed this belief (Howarth and Cooper, 1949). It has

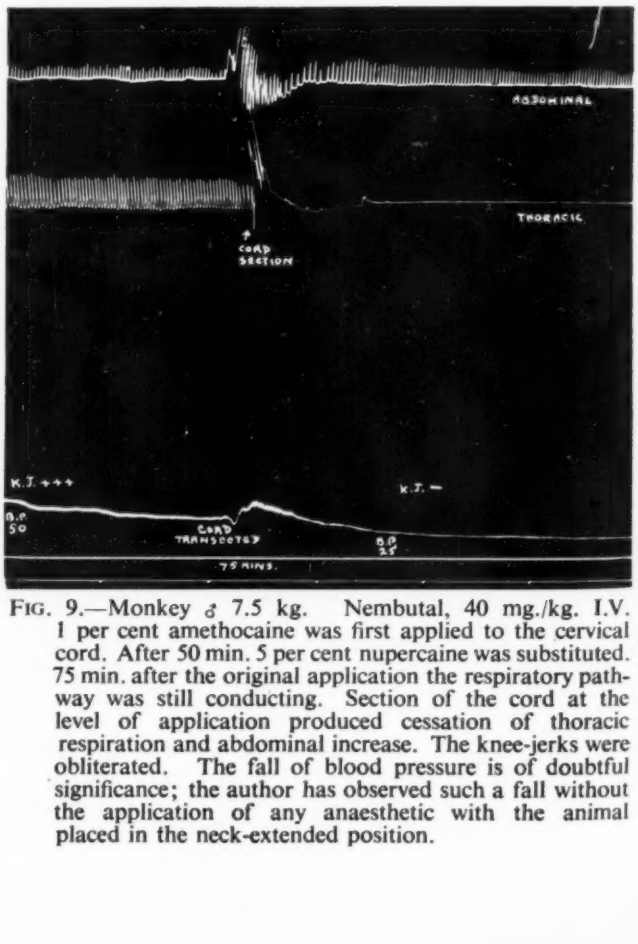

FIG. 9.—Monkey ♂ 7.5 kg. Nembutal, 40 mg./kg. I.V. 1 per cent amethocaine was first applied to the cervical cord. After 50 min. 5 per cent nupercaine was substituted. 75 min. after the original application the respiratory pathway was still conducting. Section of the cord at the level of application produced cessation of thoracic respiration and abdominal increase. The knee-jerks were obliterated. The fall of blood pressure is of doubtful significance; the author has observed such a fall without the application of any anaesthetic with the animal placed in the neck-extended position.

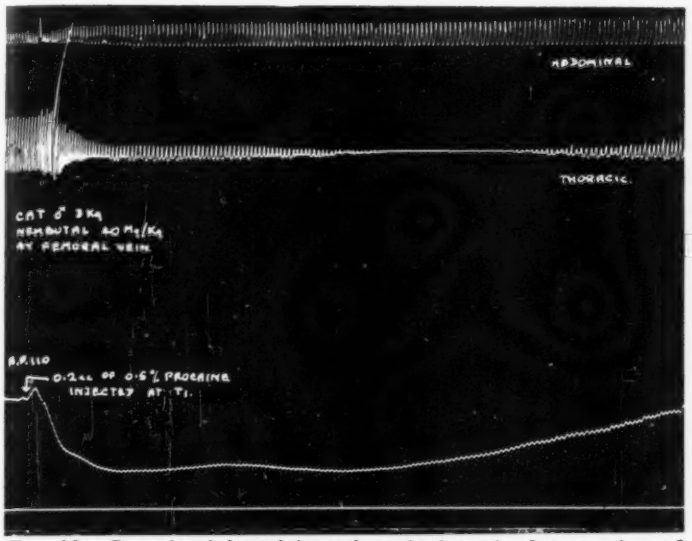

FIG. 10.—Procaine injected into the spinal cord. Interruption of descending respiratory pathway with 0.5 per cent procaine. A similar result may be obtained with a much less volume of procaine (0.05 c.c. of 2 per cent).

been established that the azygos vein is an important channel for the venous drainage of the spinal theca in the cat not only for DBP but also for compounds of sodium, phosphorus, and bromine.

DISCUSSION

From the investigations here recorded the following conclusions appear justifiable.

After introduction into the spinal theca, the concentrations of DBP, sodium, phosphorus, and bromine show a rapid reduction owing to their departure by certain channels. The substances were found in the blood stream as soon as the injection was completed (Howarth and Cooper, 1949).

As regards the anaesthetic, some is absorbed by the spinal roots which usually contain a higher concentration than that present in the C.S.F. The spinal cord too is penetrated by the anaesthetic, though the concentration within it is relatively small and is inadequate to cause a functional transection of the cord. It must not be assumed that this anaesthetic occupies the perivascular spaces, for, although Weed (1914, 1923) has shown that these spaces are in direct communication with the general subarachnoid space, it is probable that the direction of fluid flow is centrifugal rather than centripetal (Weed, 1914). Furthermore, from King's work (1939) it appears that dyes gaining access to the cord from the subarachnoid space do so by a general permeation of the pial membrane rather than by local perivascular ingress. The specific concentration in the roots supports the contention of Tuffier and Hallion (1900) that the site of action of a spinal anaesthetic is centred upon the nerve roots, a conclusion supported by Pitres and Abadie (1901). Smith and Porter (1915) assumed that the pathways of the spinal cord are interrupted, though Babcock (1925) reverted to the earlier view in accordance with the opinion of Labat (1923). Babcock did imply that the cord was slightly affected, but Graffagnino (1926–7) denied even this degree of involvement, a view endorsed by Campbell (1926), Schutz (1928), and Evans (1929). Subsequently, with the notable exception of Sebrechts (1934), there appeared a reversal of opinion by the majority of workers. Thus Koster and Kasman (1929), Russell (1929), Ferguson and North (1932), Grodinsky and Baker (1933), and Vehrs (1934) concluded that the cord is in fact penetrated by the drug. Hill and Macdonald in 1935 stated that spinal anaesthesia was generally accepted to be a root anaesthesia, but Henderson (1937) still

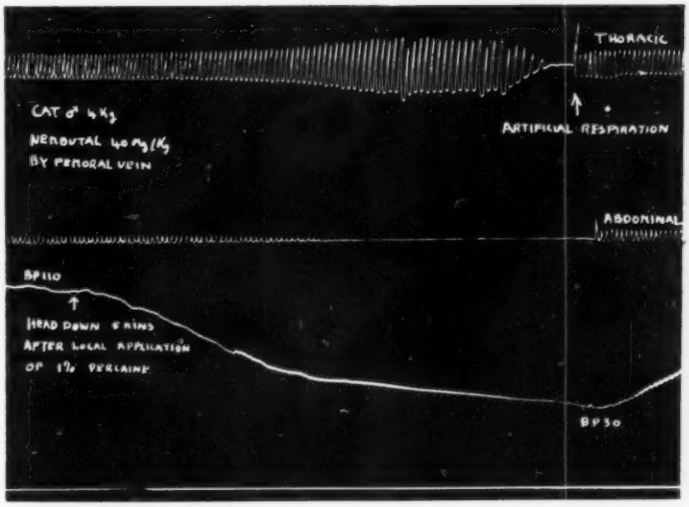

FIG. 11.—Local application 1 per cent cinchocaine to cervical cord. Head down after five minutes. Fall of B.P.; obliteration of abdominal respiration (phrenic roots): compensatory thoracic increase followed by respiratory paralysis, probably owing to arrested activity of respiratory centres. Artificial respiration, B.P. commences to rise.

adhered to anaesthetic cord block. Romberger (1941, 1943) restored the *status quo ante*, holding that the pia mater denies the drug access to the cord. This confusion seems to have arisen from the assumption that an anaesthetic present in the cord must necessarily interrupt the tracts therein. So far as the author is aware a quantitative assay of the drug within the cord has not been reported previously, and it appears that only Harrison and Frank (1932) and Kunlin (1945) have blocked the cord by direct injections of anaesthetic.

It was evident that no study of the causes of cord sequelae of spinal anaesthesia could be undertaken when the basic facts were so ill-understood. The author does not suggest, however, that some of the peripheral tracts are not blocked during spinal anaesthesia, and the fine fibres of the spinothalamic pathway may be interrupted, though he has been unable to devise a satisfactory method for the study of this phenomenon.

Some of the drug appears to ascend within the cord to the cervical regions, a route which has been suggested by Brierley and Field (1948) for phosphorus, and some may pass upwards *via* the pial venous plexus; little passes down the sciatic nerves.

A small quantity leaves the subarachnoid space *via* the lymphatic drainage, thus confirming the presence of the channel suggested by Field and Brierley (1948a and b) in their work with indian ink.

From the blood stream the drug is distributed about the body tissues, but only kidney and liver are able to concentrate it above the blood level, the kidney being far more important than the liver in this respect. The tissue/blood concentrations do not appear to show any significant variations with large changes in blood level and the time interval before autopsy. It would thus appear that tissue retention does not occur except possibly in the 20–30-hour range.

The urine provides the main pathway of removal from the body, and the concentration of the anaesthetic or its end-products is much in excess of the corresponding blood levels, a fact elicited by Burgen and Keele (1948). The blood levels themselves are consistently low. The bile forms a subsidiary excretion channel, and, though some of the products may be reabsorbed by the gut, some appear in the faeces. Some part of the drug containing benzene nuclei persists for considerable periods in the blood, and blood DBP can pass the choroid plexus. Of all the channels draining the spinal subarachnoid space, the venous route appears to be the most important (Howarth and Cooper, 1949). This is not clear in contemporary teaching.

Summary

1. A radioactive spinal anaesthetic dibromoprocaine (DBP) hydrochloride has been prepared.

2. The spinal anaesthetic properties of this drug have been investigated.

3. The distribution of the drug about the body fluids after its intrathecal injection has been studied. It has been shown that the concentration in the spinal subarachnoid space rapidly declines, associated with a rapid rise in urine concentration. The blood level remains persistently low.

4. The tissue distribution of DBP after both intrathecal and intravenous injections has been studied. After intrathecal injections only the spinal roots regularly show any capacity to concentrate the drug above the level existing in the cerebrospinal fluid at the site of injection. Of the tissues examined only kidney (and urine), liver (and bile) appear able to concentrate DBP above the circulating blood concentration. Large variations in time and dose did not produce large changes in the tissue/blood ratios.

5. It has been shown that DBP enters the spinal cord during spinal anaesthesia, though it is improbable that it is able to produce a functional cord transection.

6. Various routes of departure of this anaesthetic from the spinal theca have been studied, and, of these, that furnished by the venous system appears in the cat to be the most important.

The author wishes to thank Professor A. D. Macdonald for facilities in his laboratory and for the benefit of his advice. Also Mr. Robert West, now of A.E.R.E. Harwell, for the trouble taken in the preparation of many radioactive samples. Thanks are due also to the Medical Research Council which bore much of the financial burden of this work.

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THE EFFECT OF DIMERCAPROL ON LEAD POISONING IN MICE*

BY

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The published investigations of the effect of dimercaprol or 2 : 3-dimercaptopropanol (BAL) in lead poisoning in animals have been chiefly concerned with acute poisoning or with toxicity. Braun, Lusky, and Calvery (1946), Graham and Hood (1948), and also Germuth and Eagle (1948) reported that dimercaprol failed to protect animals poisoned with lead acetate, and that the dimercaprol-treated animals died sooner. In acute poisoning, Ginsberg and Weatherall (1948) found that dimercaprol increased the urinary excretion of lead, and altered the distribution in the organs. The present work was designed to estimate the effect of dimercaprol on the retention of lead in a more chronic form of plumbism.

METHODS

The mouse was chosen as the experimental animal because the whole animal could be ashed and a determination of the total lead content made. Lead acetate was fed with a low calcium diet to male mice in such amounts that the maximum possible daily dose was 1 mg. Pb per mouse. The procedure followed was similar to that used by Tompsett (1939), who showed that retention of lead in mice was highest on a low calcium diet. The lead acetate in most experiments was marked with a tracer of Pb^{210} (i.e., radium D). This naturally occurring isotope has the great advantage of being easily obtained from old radon tubes. The lead was fed for 8 to 14 days, and after varying intervals of time the animals were killed, the stomach and intestine removed and discarded, to remove any lead excreted into the gut, and the whole carcass ashed; the ash was brought into solution with the minimum of acid, and made up to 100 ml. with water. Lead was then estimated chemically in aliquots of the ash solution by the method described previously (Tompsett and Anderson, 1935). Where radioactive lead had been administered, the activity of the ash solution was estimated by means of a Geiger-Müller counter. In practice the β radiation of radium D is too weak to count and the stronger β radiation of radium E in equilibrium with radium D is counted. Ash samples had therefore to be kept until the mixture reached equilibrium, a matter of 30–40 days.

Dimercaprol dissolved in oil, or as a freshly prepared solution in saline, was injected subcutaneously in daily doses of 50 mg./kg. mouse, either during the entire period of administration of lead or in the subsequent period.

RESULTS

The results obtained when dimercaprol was given simultaneously with the lead are shown in Table I. The first two experimental animals received dimercaprol in oil solution daily for eight days, the other three dimercaprol in saline for eleven days. The results of the chemical analysis show that the lead content of the mice receiving dimercaprol was significantly lower than that of the controls. There is not a very close agreement

TABLE I

ADMINISTRATION OF LEAD AND DIMERCAPROL (BAL) SIMULTANEOUSLY

Lead content of mice in mg./100 g. *a* by count. *b* by chemical analysis

Controls		Dimercaprol		
a	*b*	*a*	*b*	
1.0	1.08	0.39	0.31	Lead 10 days
0.96	1.20	0.63	0.56	BAL in oil 8 days
0.75	0.73	0.48	0.59	Lead 14 days
1.06	1.11	0.71	0.55	BAL in saline 11 days
[1.92]	0.89	0.40	0.42	
Mean: 0.94	1.00	0.52	0.48	
Range:	0.73–1.11		0.31–0.59	
S.D.:	±0.19		±0.12	

* The substance of a paper read to the first International Congress of Biochemistry at Cambridge, August, 1949.

† Present address.

between the figures obtained by count and those by chemical analysis, but they are of the same order and serve as a useful check. For some unexplained reason one of the controls, in brackets, gave a very high count, and has been excluded from the calculation of the mean. The lead content is given here as mg./100 g., but as the dead weights were all of the same order the same conclusions are reached if one compares mg. of lead per mouse.

The lead given was approaching a lethal dose, as in some similar experiments one or more of the animals died during administration of the lead. When mice which had been fed lead previously were treated with dimercaprol the results shown in Table II were obtained. The first two experimental animals received dimercaprol in oil, and the next six dimercaprol in saline. This second series were fed non-radioactive lead. Three of the control animals and four of the treated died near the end of the experiment on the days shown. Two animals died before dimercaprol treatment was started and were not analysed. Treatment with dimercaprol had no significant effect on the final lead content. The figures for the second series show a wide variation owing to the variation in the dead weights in this series where older animals were used (the previous animals were all "20 g. mice"). If the actual amounts of lead found in the individual mice, as shown in Table III, are compared, it will be seen that the variation is less, and that dimercaprol had no significant effect.

TABLE II

ADMINISTRATION OF LEAD FIRST THEN DIMERCAPROL (BAL)

Lead content of mice in mg./100 g. *a* by count. *b* by chemical analysis

Controls		Dimercaprol		
a	*b*	*a*	*b*	
1.58	0.72	1.17	0.92	Lead 8 days then 7 days
0.98	1.08	0.89	1.04	BAL in oil last 4 days
Mean:	0.90		0.98	
	0.53		1.15 (17)	
	0.95 (20)		0.95 (21)	Lead 10 days
	0.41		0.85 (20)	then BAL in
	0.45		0.50 (22)	saline 12 days
	— (12)		0.61	
	— (12)		0.47	
Mean:	0.58		0.75	

Numbers in parentheses indicate day of death.

TABLE III

LEAD FIRST THEN DIMERCAPROL

Actual lead content of mice in mg. from Table II. Chemical analysis

Controls	Dimercaprol	
0.18	0.23	Lead 8 days then 7 days
0.26	0.27	BAL last 4
0.18	0.22	
0.19	0.24	Lead 10 days
0.16	0.21	then BAL for
0.18	0.15	12 days
—	0.19	
—	0.16	
Mean: 0.19	0.21	
Range: 0.16–0.26	0.15–0.27	
S.D.: ±0.035	±0.038	

DISCUSSION

Dimercaprol did not diminish the toxicity and more animals died in the experimental series than in the control. Germuth and Eagle (1948) have reported that the lead-dimercaprol complex is almost as toxic as lead itself.

Dimercaprol given during the exposure to lead caused less lead to be accumulated in the body, but how this was brought about, whether by an increase in excretion or by a decrease in absorption or by both cannot be determined from the evidence presented here. There is some published evidence that dimercaprol produces a temporary increase in urinary excretion of lead both in animals (Germuth and Eagle, 1948; Ginsberg and Weatherall, 1948) and in man (Ryder, Cholak, and Kehoe, 1947; Telfer, 1947).

The fact that dimercaprol had no effect on the loss of lead from the animals previously treated with lead for eight to ten days provides no support for its use in the treatment of chronic lead poisoning.

SUMMARY

1. Lead acetate marked with a tracer of Pb^{210} (radium D) was fed with a low calcium diet to mice, and after varying periods the lead content of the whole animal was determined by chemical analysis and count of β radiation.

2. The lead content of mice which had received 50 mg. dimercaprol per kg. daily, and lead simultaneously for 10 to 14 days, averaged 0.48 mg.

Pb/100 g. of mouse, and was significantly lower than that of controls receiving lead alone, which averaged 1.0 mg. Pb/100 g.

3. When lead alone was administered for eight to ten days, subsequent treatment with dimercaprol during a recovery period of one to two weeks had no significant effect on the final lead content.

4. These results provide no indications for the use of dimercaprol in treatment of chronic lead poisoning.

I am indebted to Dr. Rowlands and to present members of the physics department of St. Mary's Hospital Medical School for advice and assistance with the estimation of the radio-activity. I am also indebted to Sir Jack Drummond and Messrs. Boots for a supply of dimercaprol.

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THE EFFECTS OF DIMERCAPROL AND PARATHYROID EXTRACT ON THE SUBACUTE DISTRIBUTION OF LEAD (ACETATE) IN RABBITS

BY

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It has been shown that dimercaprol (2 : 3-dimercaptopropanol, BAL) increases the excretion and alters the distribution of lead in rabbits when the dimercaprol is given less than twenty-four hours after intravenous administration of lead acetate (Ginsburg and Weatherall, 1948). This paper extends these observations to a later phase in the distribution of a single intravenous dose of lead. At this phase most of the lead remaining in the body was found in the bones. It seemed likely that dimercaprol would not have much effect on lead so deposited, and, at the suggestion of Professor J. H. Gaddum, the effect of parathyroid extract, which is believed to mobilize lead from bones (Hunter and Aub, 1927), was also examined, alone and in conjunction with dimercaprol.

METHODS

The procedure in these experiments followed that of Ginsburg and Weatherall (1948) with some modifications, as follows: Pb^{210} (radium D) was used as a tracer instead of Pb^{212} (thorium B), the short half-life of which made it useless for the present purpose. Radium E and radium F were removed from an equilibrium mixture of radium D, E, and F by displacement with nickel, added as foil to a solution in hydrochloric acid. A small quantity of the resulting solution of radium D and some dextrose were added to a solution of lead acetate so that the final solution contained 2.07 mg. Pb and 2–3 microcuries of radium D per ml. in 4 per cent (w/v) dextrose. Doses of 1.0 ml./kg. (i.e., 0.01 mM/kg.) were administered to rabbits of both sexes and various breeds by injection into the marginal vein of one ear at a rate of 2.0 ml. per min. After injection the rabbits were placed in metabolism cages and allowed food and water *ad libitum* from vessels placed outside windows in the cages so that spilt food or water did not dilute the urine nor contaminate the faeces. Faeces and urine were collected at first every second or third day, and later, during the period of treatment, daily. The animals were killed by a blow on the occiput 13 or 21 days after the injection of lead acetate. Solutions of dimercaprol for injection were freshly prepared in 66 per cent (v/v) aqueous propylene glycol. Both dimercaprol and parathyroid extract ("Parathormone" Lilly) were injected into the paravertebral muscles in doses as indicated below.

Ginsburg and Weatherall's (1948) procedure for taking and ashing samples of tissue was followed without modification. Samples of liver, epiphysis and diaphysis were routinely, and of bone marrow and kidney were sometimes, made in duplicate. Estimates of the concentration in other tissues were based on single samples. Before estimating their radioactivity, samples were allowed to stand for at least five weeks to allow an equilibrium mixture of radium D and E to form, because the counter used was not sufficiently sensitive to detect the low energy β-rays, emitted by the radium D, but counted the more energetic emission from radium E. The samples were diluted to a known volume, usually 10, 25, or 50 ml., and their activity was measured by means of an M.R.C. type 1 fluid Geiger-Müller counter. The total count per minute, corrected for the background count, with the counter used was linearly related to the amount of radioactive material present and was negligibly affected by variations in the density of the solution within the range involved in these experiments. The concentration and quantity of lead in tissues was calculated as before, except that the amount of lead in bone was assessed from the mean of the values in all the types of bone sampled, for reasons discussed below.

The standard error of the lead estimations, calculated from duplicate determinations made during the experiments, was ± 12.3 per cent. The total amount of lead accounted for in the entire animal and its excreta averaged 78 per cent of the dose, with a standard deviation of ± 12 per cent. The discrepancy between duplicate determinations is larger than in the earlier work, mainly if not entirely because much

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smaller quantities of radioactive tracer were used and the time of counting samples was not increased sufficiently to attain the same accuracy. The average fraction of the dose accounted for (78 instead of 90 per cent) is rather lower. The calculation of the total amount of lead in bone involves a particularly crude approximation, and in these experiments such a faulty approximation had a far larger effect than in the acute experiments where only 5 instead of 25 per cent of the dose was involved. The poorer recovery is clearly less satisfactory, but in the circumstances it does not appear to be so poor as grossly to invalidate the results obtained.

Results

Results are presented for the distribution and excretion of lead in nine rabbits, all of which received, by intravenous injection, a single dose of lead acetate containing 2.07 mg. of lead per kg. of body weight. Some of the rabbits were treated with dimercaprol or with parathyroid extract or with both, as indicated in Table III, and all were killed after thirteen or twenty-one days. Rabbits receiving no dimercaprol received instead similar injections of propylene glycol (66 per cent v/v in water). The parathyroid extract was given in the morning. The dimercaprol was given at the same time, though injected at a different site, and again four hours later, in order to cover the period in which the greatest mobilization of lead might be expected. The experiments were conducted in three groups, one of which consisted of rabbits 377 and 379, one of 378, 380, and 384, and one of 396, 397, 398, and 399. All the rabbits within a group were treated at the same time, and comparisons between rabbits in the same group are therefore less subject to incidental sources of variations than are comparisons between rabbits in different groups.

The excretion of lead before treatment was started is shown in Table I. It has been shown (Ginsburg and Weatherall, 1948) that, within twenty-four hours of giving lead, excretion occurs chiefly in the urine. In the present experiments, over a longer period, faecal excretion was usually two to three times greater. The amounts excreted appeared to depend to some extent on the quantity of excreta passed.

TABLE I

THE EXCRETION OF LEAD BY RABBITS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. PB/KG.), BEFORE ANY FURTHER TREATMENT

Excreta were collected at 10 a.m. The period "Days 0–3" therefore ends at 10 a.m. on the third day after injection and the period "Days 3–5" runs from 10 a.m. on the third day to 10 a.m. on the fifth day. The excreta of rabbits 378, 380, and 384 were collected and estimated in bulk during the first fourteen days and so are not included in this table.

Rabbit No.:	Total quantity of excreta and concentration of lead in excreta											
	379*		377		398		396		397		399	
URINE	ml.	μg./ml.	ml.	μg./ml.	ml.	μg./ml.	ml.	μg./ml.	ml.	μg./ml.	ml.	μg./ml.
Days 0–3 ...	162	0.44	194	0.50	220	0.99	280	1.27	131	2.36	165	0.97
,, 3–5 ...	178	0.16	286	0.19	97	0.82	192	0.57	38	1.41	67	0.63
,, 5–7 ...	42	0.37	56	0.17	198	0.29	182	0.38	126	0.71	246	0.33
,, 7–9 ...	36	0.42	—	—								
,, 9–11...	112	0.15	—	—	60	0.23	146	0.38	99	0.37	90	0.30
,, 11–13...	102	0.22	—	—	184	0.40	180	0.23	24	0.69	137	0.42
,, 13–14...	—	—	—	—	73	0.17	52	0.20	56	0.31	45	0.36
FAECES	g.	μg./g.	g.	μg./g.	g.	μg./g.	g.	μg./g.	g.	μg./g.	g.	μg./g.
Days 0–3 ...	125	2.58	35	3.37	43	11.02	70	2.34	60	7.21	65	1.70
,, 3–5 ...	26	3.63	18	4.97	119	12.56	83	2.60	31	3.45	86	1.84
,, 5–7 ...	61	2.93	30	3.51	37	1.61	103	1.52	42	6.24	97	0.80
,, 7–9 ...	71	0.31	—	—	145	1.16	142	0.56	155	2.23	125	0.16
,, 9–11...	124	0.34	—	—								
,, 11–13...	40	0.17	—	—	155	0.58	100	0.40	181	0.98	89	0.63
,, 13–14...	—	—	—	—	68	0.13	45	0.63	42	0.89	50	0.49
Total percentage of the dose excreted in 14 days												
Urine ...	—		—		10.8		16.9		12.3		9.2	
Faeces ...	—		—		36.1		17.1		32.7		8.0	

* Treated with propylene glycol on days 8–11.

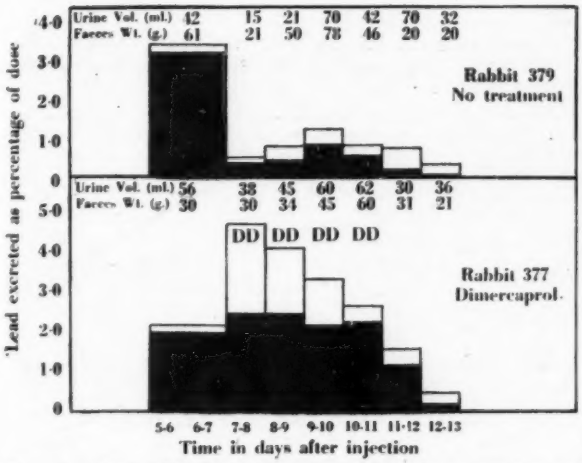
Urine Vol. (ml.) 42 15 21 70 42 70 32
Faeces Wt. (g.) 61 21 50 78 46 20 20
Rabbit 379
No treatment
Urine Vol. (ml.) 56 38 45 60 62 30 36
Faeces Wt. (g.) 30 30 34 45 60 31 21
DD DD DD DD
Rabbit 377
Dimercaprol
Lead excreted as percentage of dose
4·0
3·0
2·0
1·0
0
5·0
5-6 6-7 7-8 8-9 9-10 10-11 11-12 12-13
Time in days after injection

FIG. 1.—The effect of dimercaprol on the excretion of lead in the urine and faeces 8–11 days after the intravenous administration of lead acetate (2.07 mg. Pb/kg.). Ordinates: Amount of lead excreted as a percentage of the dose administered (1 per cent = 20.7 μg. Pb/kg. body weight). Abscissae: Number of days after the injection of lead acetate. D = 12.5 mg./kg. dimercaprol injected intramuscularly. ▭ Urinary excretion. ■ Faecal excretion.

The effects of the treatments on the elimination of lead in the urine and faeces are shown in Figs. 1 and 2. When dimercaprol was administered seven days after the lead (377), a tenfold increase in the urinary excretion of lead occurred during the first day of treatment, after which the amount fell progressively during subsequent days of treatment. The faecal lead also increased, although probably not significantly. In the rabbits treated with dimercaprol on the fifteenth and subsequent days after the administration of lead (378 and 396) there was an increase in the concentration of lead in the urine and faeces. This was accompanied by a diminution in the quantity of excreta, so that there was no increase in the total amount of lead excreted. A slight fall of doubtful significance occurred in the total amount of lead excreted by the rabbits treated with parathyroid extract or with dimercaprol and parathyroid extract, probably owing to the diminution in the quantity of excreta which also occurred in these rabbits. Rabbit 399 developed acute retention of urine during the days preceding death, when over 400 ml. of urine was found in the bladder. No cause for this retention was apparent, but it accounts for the large amount of lead recorded as excreted on the twenty-first day.

TABLE II

THE CONCENTRATION OF LEAD IN THE BLOOD CELLS OF RABBITS POISONED WITH LEAD ACETATE DURING TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT

	μg. Lead per g. of blood cells		
Rabbit No.	378	384	380
Treatment (details as in Table III) ...	Dimercaprol	Parathyroid extract	Dimercaprol and parathyroid extract
Immediately before treatment	0.16	0.50	0.18
3 hours after first dose	<0.05	0.26	0.07
24 hours after first dose	0.09	0.20	<0.07
3 days after end of treatment (i.e., at death)	0.19	0.21	0.07

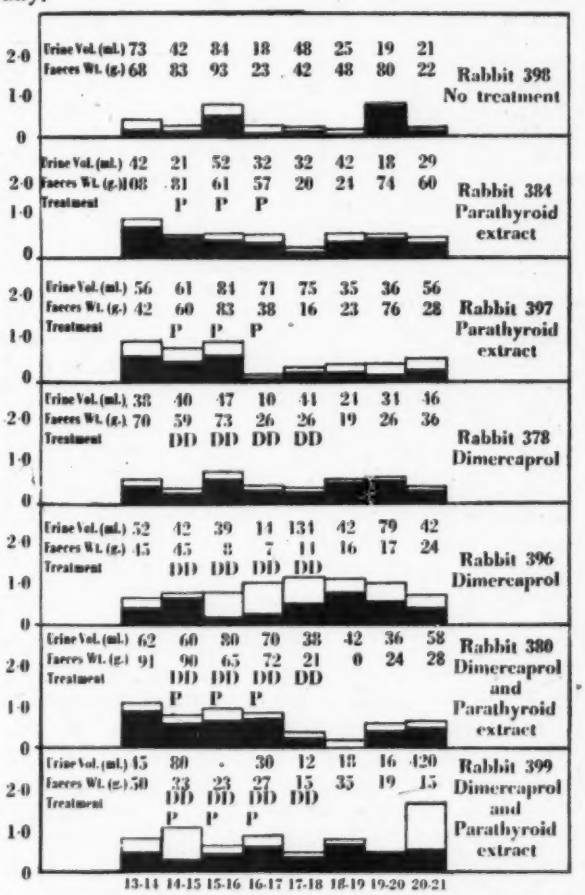
Urine Vol. (ml.) 73 42 84 18 48 25 19 21
Faeces Wt. (g.) 68 83 93 23 42 48 80 22
Rabbit 398
No treatment
Urine Vol. (ml.) 42 21 52 32 32 42 18 29
Faeces Wt. (g.) 108 81 61 57 20 24 74 60
Treatment P P P
Rabbit 384
Parathyroid extract
Urine Vol. (ml.) 56 61 84 71 75 35 36 56
Faeces Wt. (g.) 42 60 83 38 16 23 76 28
Treatment P P P
Rabbit 397
Parathyroid extract
Urine Vol. (ml.) 38 40 47 10 44 21 31 46
Faeces Wt. (g.) 70 59 73 26 26 19 26 36
Treatment DD DD DD DD
Rabbit 378
Dimercaprol
Urine Vol. (ml.) 52 42 39 14 134 42 79 42
Faeces Wt. (g.) 45 45 8 7 11 16 17 24
Treatment DD DD DD DD
Rabbit 396
Dimercaprol
Urine Vol. (ml.) 62 60 80 70 38 42 36 58
Faeces Wt. (g.) 91 90 65 72 21 0 24 28
Treatment DD DD DD DD P P P
Rabbit 380
Dimercaprol and Parathyroid extract
Urine Vol. (ml.) 45 80 - 30 12 18 16 420
Faeces Wt. (g.) 50 33 23 27 15 35 19 15
Treatment DD DD DD DD P P P
Rabbit 399
Dimercaprol and Parathyroid extract
2·0
1·0
0
13-14 14-15 15-16 16-17 17-18 18-19 19-20 20-21
Time in days after injection

FIG. 2.—The effect of dimercaprol and parathyroid extract on the excretion of lead in the urine and faeces 15–18 days after the intravenous administration of lead acetate (2.07 mg. Pb/kg.). Ordinates, abscissae, and symbols as in Fig. 1. P = 8 units/kg. parathyroid extract injected intramuscularly.

TABLE III

THE EFFECT OF TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT ON THE CONCENTRATION OF LEAD IN THE TISSUES OF RABBITS THIRTEEN AND TWENTY-ONE DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. PB/KG.)

	Microgrammes of lead per gramme fresh weight of tissue								
Time after giving lead ...	13 days		21 days						
Treatment with parathyroid extract	None	None	None	None		8 u./kg./day on days 15, 16, and 17		8 u./kg./day on days 15, 16, and 17	
Treatment with dimercaprol ...	None	12.5 mg./kg. twice daily, days 8–11	None	12.5 mg./kg. twice daily, days 15–18		None		12.5 mg./kg. twice daily, days 15–18	
Rabbit No. ...	379♀	377♂	398♂	378♀	396♂	384♂	397♀	380♀	399♂
Init. wt., kg. ...	1.40	1.50	2.02	1.90	1.82	1.70	2.06	1.90	2.02
Final wt., kg. ...	—	—	1.90	1.60	1.65	1.80	1.80	1.80	—
Plasma	0.06	0.02	<0.16	<0.09	<0.22	<0.10	<0.13	<0.05	<0.16
Blood cells ...	2.25	0.72	0.62	0.19	0.34	0.21	<0.27	0.07	0.35
Spleen	—	3.44	<0.64	0.30	<1.30	<0.90	<0.83	<0.23	<1.50
Bone marrow ...	9.8	6.0	3.50	<0.25	7.0	4.6	4.2	1.10	2.70
Liver	3.0	4.1	1.9	0.62	2.0	3.3	2.2	4.7	1.4
Bile	<1.54	0.87	<0.64	1.0	<2.9	0.33	<1.0	0.59	<2.21
Pancreas ...	<0.17	0.19	—	<0.08	—	<0.45	—	<0.15	—
Colon	0.07	0.14	<0.07	0.01	<0.10	0.03	<0.08	0.01	<0.12
Colon contents ...	0.34	0.29	<0.30	0.03	<0.12	0.04	<0.15	0.11	—
Kidneys	0.63	1.68	0.67	0.16	0.45	0.53	0.55	0.27	0.50
Adrenals ...	<1.45	<0.48	—	<1.14	—	<2.07	—	<0.87	—
Gonads	<0.95	0.50	—	<0.64	—	<0.64	—	<0.55	—
Seminal vesicles...	—	—	—	—	—	<0.11	—	—	—
Uterus	<0.16	—	—	<0.04	—	—	—	<0.06	—
Lungs	0.42	0.14	<0.13	0.83	1.86	0.02	0.34	<0.02	0.20
Skeletal muscle ...	0.02	0.05	<0.09	<0.01	<0.09	<0.02	0.13	<0.01	<0.07
Diaphysis ...	9.0	5.3	5.1	3.8	2.6	7.0	3.5	4.4	1.4
Epiphysis ...	9.6	12.6	12.9	5.7	8.0	10.9	5.2	3.5	2.5
Ribs	7.6	10.3	19.0	6.7	9.7	10.0	7.8	6.4	1.5
Vertebrae ...	11.9	11.5	—	6.2	—	10.0	—	5.4	—
Skull vault ...	9.8	15.0	12.8	10.4	5.5	7.8	5.0	12.5	2.6
Brain	<0.09	0.17	<0.13	0.12	<0.12	0.10	0.15	<0.02	0.11
Skin	0.12	0.10	0.20	0.11	<0.15	0.10	<0.14	0.10	0.30

The effects of the treatments on the concentration of lead in the blood cells are shown (for three rabbits) in Table II. After the first treatment with dimercaprol, or parathyroid extract, or both, the concentrations of lead in the blood cells fell by one-half to two-thirds. They remained low during treatment and afterwards, if anything, tended to rise. The counts were very low and were prolonged to give an accuracy of only ±25 per cent, so that even the initial fall is barely significant. The corresponding samples of plasma contained no lead, or too little to be detected—i.e., less than 0.3–0.4 microgramme per gramme in the samples taken before death and less than 0.1 microgramme per gramme in the samples taken at death.

The concentration of lead in microgrammes per gramme of wet weight of tissue and the percentage of the dose found at death (i.e., three days after the end of treatment) in various organs and calculated for various tissues are shown in Tables III, IV, and V. In the previous paper in this series, Ginsburg and Weatherall (1948) calculated the amount of lead in bone from the concentration in diaphyses, because the concentration of lead in the bone marrow was much higher and it was uncertain to what extent the marrow present in the interstices of the cancellous bone of the samples of epiphyses was raising the concentration there. In the present experiments the concentration of lead in the epiphyses was much higher than the concentration in either marrow or diaphyses, and

TABLE IV

THE EFFECT OF TREATMENT WITH DIMERCAPROL AND PARATHYROID EXTRACT ON THE DISTRIBUTION OF LEAD IN RABBITS THIRTEEN AND TWENTY-ONE DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. PB/KG.)

	Percentage of dose in entire organ or tissue								
Time after giving lead ...	13 Days		21 Days						
Treatment with parathyroid extract	None	None	None	None		8 u./kg./day on days 15, 16, and 17		8 u./kg./day on days 15, 16, and 17	
Treatment with dimercaprol ...	None	12.5 mg./kg. twice daily, days 8–11	None	12.5 mg./kg. twice daily, days 15–18		None		12.5 mg./kg. twice daily, days 15–18	
Rabbit No. ...	379♀	377♂	398♂	378♀	396♂	384♂	397♀	380♀	399♂
Init. wt., kg. ...	1.40	1.50	2.02	1.90	1.82	1.70	2.06	1.90	2.02
Final wt., kg. ...	—	—	1.90	1.60	1.65	1.80	1.80	1.80	—
Plasma	0.12	0.06	<0.30	<0.24	<0.45	<0.22	<0.30	<0.11	<0.31
Blood cells ...	3.11	0.62	0.86	0.20	0.45	0.24	<0.30	0.08	0.48
Spleen	—	0.12	<0.02	0.01	0.02	<0.02	0.02	<0.01	<0.02
Bone marrow ...	9.6	5.8	3.3	<0.38	6.8	4.4	4.0	0.99	2.5
Liver	3.8	6.0	2.2	0.70	2.0	4.5	2.8	5.4	1.3
Pancreas ...	<0.01	0.03	—	<0.01	—	<0.01	—	<0.01	—
Colon	0.03	0.07	<0.02	0.01	<0.02	0.01	<0.02	0.01	<0.02
Colon contents ...	0.18	0.12	<0.04	0.01	<0.05	0.01	<0.04	0.04	—
Kidneys	0.24	0.55	0.18	0.04	0.14	0.16	0.13	0.07	0.14
Adrenals ...	<0.02	<0.01	—	<0.01	—	<0.06	—	<0.01	—
Gonads	<0.01	0.06	—	<0.01	—	<0.02	—	<0.01	—
Lungs	0.08	0.03	<0.02	0.02	0.26	0.01	0.05	<0.01	0.03
Skeletal muscle ...	0.50	1.25	<2.2	<0.68	<2.3	<0.58	3.3	<0.28	<1.8
Bone	26.0	29.2	35.8	19.2	18.7	27.4	15.7	18.7	5.7
Brain	<0.02	0.04	<0.02	0.02	<0.02	0.02	0.03	<0.01	0.02
Skin	0.70	0.52	1.2	0.61	<0.87	0.60	<1.00	0.57	1.8
Injected ear ...	3.86	15.2	0.23	0.02	0.14	0.41	0.02	0.59	33.3
Total in carcass†	50%	63%	44%	21%	29%	39%	26%	26%	45%
Excreted:									
Urine, days 0–14	5.9*	(10.7)*	10.8	11.3	16.9	11.8	12.3	14.9	9.2
Urine, days 14–21	—	—	1.1	0.8	3.2	0.8	1.7	1.3	3.1
Faeces, days 0–14	23.0*	(19.3)*	36.1	33.9	17.1	28.9	32.7	27.1	8.0
Faeces, days 14–21	—	—	5.7	2.7	3.6	2.7	2.0	3.1	4.9
Total excreted ...	29%	30%	54%	49%	41%	44%	49%	45%	25%
Total accounted†	79%	93%	98%	70%	70%	83%	75%	71%	70%

* Days 0–13. † Including items shown in Table V.

clearly a larger part of the entire dose had been taken up at the ends of the bones. The total amount of lead in bone has therefore been estimated from the mean concentration in all the types of bone consistently sampled, without weighting for the relative proportions of the different types. Large inaccuracies are probably so introduced, and the figures are useful only as a rough check that a reasonable fraction of the entire dose has been accounted for.

In the first pair of rabbits (377 and 379), the concentration of lead in the wall of different parts of the alimentary canal above the rectum was fairly uniform, and therefore in subsequent experiments only one portion of the intestine, the colon, was sampled. The rest of the alimentary canal was the largest amount of tissue not examined in the later animals; but probably did not include more than 1 or 2 per cent of the dose.

TABLE V

THE CONCENTRATION OF LEAD AND THE PERCENTAGE OF THE DOSE OF LEAD IN CERTAIN ADDITIONAL TISSUES OF RABBITS THIRTEEN DAYS AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 MG. PB/KG.) WITHOUT AND WITH TREATMENT WITH DIMERCAPROL

	μg. Lead per g. fresh weight of tissue		Percentage of dose in entire organ or tissue	
Rabbit No. ...	379	377	379	377
Treatment ...	Propylene glycol	Dimercaprol	Propylene glycol	Dimercaprol
Stomach ...	0.07	0.18	0.06	0.10
Stomach contents ...	0.07	0.07	0.11	0.16
Small intestine	<0.07	0.14	<0.10	0.10
Small intestine contents ...	0.14	0.02	0.04	0.02
Caecum ...	0.11	0.22	0.13	0.53
Caecum contents ...	0.27	0.34	0.63	2.25
Rectum ...	0.04	0.15	0.01	0.04
Rectum contents ...	0.66	0.32	0.44	0.11
Heart ...	<0.18	<0.15	<0.02	<0.02
Bladder ...	1.18	<0.43	0.05	<0.02
Spinal cord...	0.38	0.67	0.05	0.09
Eyes... ...	<0.12	<0.15	<0.03	<0.03

Of the lead which remained in the body, regardless of treatment and apart from that which had not been injected cleanly and remained at the site of injection (notably in rabbits 377 and 399), 50 to 95 per cent was found in the bones. No other single tissue accounted for more than a few per cent of the dose, and only the liver and bone marrow contained more than 2 per cent consistently. Similarly the highest concentrations were found in the bones, bone marrow, and liver, which generally contained 2–15 microgrammes of lead per gramme of tissue. Other tissues rarely contained more than 1 microgramme per gramme. The concentration in blood cells, lungs, kidneys, bile from the gall bladder, and skin tended to be above 0.1 microgramme per gramme, whereas those in skeletal muscle, the alimentary canal, and brain tended to be below this level.

General inspection of the results shows no striking differences between differently treated animals. The concentrations in bone were somewhat higher in the untreated rabbits than in any others. Interpretation of this difference is confused by the great variability of the concentration in different bones, by the large deposit of lead at the site of injection in one rabbit (399), and by variation in the amount of lead excreted before treatment was started. The total amount of lead accounted for tended to be low when the concentration of lead in the bones was low; that is, as here calculated, there was no completely corresponding increase in the amount of lead outside the skeleton in the treated animals, and the evidence that appreciable quantities of lead are removed from the bone by any of the treatments is unconvincing. Other tissues showed no consistent differences which could be related to the treatments.

Discussion

The distribution of lead in the untreated rabbits of this series showed no unexpected features. As has generally been found in rabbits (Kisskalt and Friedmann, 1914; Lomholt, 1924; Kehoe and Thamann, 1933), the excretion of lead, even after parenteral administration, was greater in the faeces than in the urine after the first day or two, and the combined excretion amounted to about half the total dose in three weeks. The rate of excretion roughly followed an exponential curve, and had reached a very low level at the end of these experiments. Even if there was no further decrease in the rate of excretion after three weeks, it would have taken a period of the order of a hundred days to excrete the rest of the lead in the body. This residual lead was, as expected, mainly in the bones; and the bone marrow and liver were the only other tissues in which concentrations usually exceeded 1 microgramme per gramme. Little attention has hitherto been paid to the bone marrow as distinct from bone (cf. Ginsburg and Weatherall, 1948), and the persistence of lead in the marrow is clearly interesting in relation to the mechanism by which lead produces anaemia. The present figures for the concentration in muscle are lower than those of Kisskalt and Friedmann (1914) and of Kehoe and Thamann (1933), even allowing for the difference in dosage: but when the small quantities of lead involved are considered, the differences are not striking nor surprising. In the central nervous system the discrepancies are larger. Kehoe and Thamann's method was unreliable (Kehoe, Thamann, and Cholak, 1935) and their figures are very variable and may be disregarded. Kisskalt and Friedmann consistently found concentrations several hundredfold higher than those reported here. With quantities of the order involved, the reliability of their method is perhaps questionable. In more acute poisoning, Weyrauch (1931) failed to detect any lead in the brain of rabbits sixteen hours after the intravenous injection of lead nitrate (12 mg. Pb/kg.), though the sensitivity of his method was not sufficient to exclude concentrations of the order quoted here: and Ginsburg and

Weatherall (1948) found slightly higher figures at six and twenty-four hours after injection than the present ones. On the whole it appears unlikely that concentrations of lead much exceeding 0.1 microgramme per gramme of tissue occur in the brains of rabbits after the intravenous injection of salts of lead.

The effects of treatment were small. Eight days after the injection of lead acetate, dimercaprol increased the urinary excretion so that in all about an extra 4 per cent of the dose was excreted Fifteen days after the injection, the effect of dimercaprol, if any, did not exceed 2 per cent of the dose. Germuth and Eagle (1948) observed larger increments in the urinary excretion of lead by rabbits up to eleven days after the last dose of lead, but they had administered several doses of 200 mg. lead acetate subcutaneously and their rabbits therefore had a depot from which lead was almost certainly being continuously absorbed and was in addition possibly mobilized by dimercaprol. Even so, as here, successive doses of dimercaprol had a rapidly diminishing effect on urinary excretion, and any increase in faecal excretion was obscured by a diminished output of faeces. During treatment the amount of lead in the blood cells decreased, and three days after treatment the concentration of lead in the cells was highest in the untreated animal of each set. The difference in concentrations corresponded to a difference of about 2.5 per cent of the dose in the shorter experiments and about 0.5 per cent in the longer ones, and so was not far from accounting for all the extra lead excreted. As indicated above, the bones may also have contributed a little. A decreased concentration of lead in the bones of rabbits poisoned with large amounts of lead and treated with dimercaprol has been reported by Lusky, Braun, and Laug (1948), but no quantitative details were given. In the present experiments the variation between identically treated rabbits was generally larger than any changes attributable to treatment, and so attempts precisely to account for the small movements of lead which may have occurred are unprofitable. The data of Ryder, Cholak, and Kehoe (1947) and of Telfer (1947) suggest that dimercaprol has rather more effect on the lead of the blood cells and urine in man than in rabbits.

The changes after parathyroid extract were also minimal. There was no increase in the excretion of lead and, if anything, a reduction in the concentration of lead in the blood cells. It does not appear that, with the doses used in the rabbit, parathyroid extract has any appreciable effect on the distribution of lead once most of the lead is deposited in the bones. Consequently the rationale of using dimercaprol and parathyroid extract together was not fulfilled, and the negative results of the combination must be attributed to the lack of action of the parathyroid extract alone. This lack of action was a little surprising. However, the literature about the action of parathyroid extracts on lead metabolism is conflicting. An increase in the concentration of lead in the blood of rabbits (Teisinger, Joachim, and Kodicek, 1938) and of man (Schmitt and Taeger, 1937) has been reported, but an increase in that of the latter has not been confirmed (Teisinger, 1938), and the rise in blood lead in Schmitt and Taeger's patient was accompanied by far less increased excretion of lead than in the cases observed by Hunter and Aub (1927). Almost any interpretation can be put on these findings, and we have failed to find in the literature any complete account of the influence of parathyroid extracts on the distribution of lead in experimental animals. Further observations are clearly desirable to establish whether in any circumstances parathyroid extracts really effect the mobilization of lead from bones.

Summary

1. The distribution of lead in the tissues of rabbits thirteen and twenty-one days after the intravenous injection of lead acetate (2.07 mg. Pb/kg.) has been studied by use of the isotope Pb^{210} (radium D). Some of the rabbits were treated with dimercaprol, or parathyroid extract, or both, for some days during the week before they were killed.

2. Apart from a transient increase in the urinary excretion of lead after dimercaprol, none of the treatments caused any substantial change in the distribution or the excretion of lead.

3. About 50 per cent of the dose of lead was excreted in twenty-one days, predominantly in the faeces. The bones contained about 25 per cent of the dose twenty-one days after injection. The bone marrow and the liver were the only other tissues which consistently contained more than 1 per cent.

4. Treatment with dimercaprol and parathyroid extract appears to have no useful effect in rabbits subacutely poisoned with lead, but confirmation of this finding in other species is desirable.

We wish to thank Dr. N. Miller for much helpful advice during these experiments, and Mrs. Irene Bell for careful technical assistance. We are grateful also to the Medical Research Council for a training grant to K.R.A. and for an expenses grant to M.W., and to the Therapeutic Research Corporation for a personal and expenses grant to M.G.

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THE ANTAGONISM OF CURARIZING ACTIVITY BY PHENOLIC SUBSTANCES *

BY

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In the course of the biological assay of solutions of *d*-tubocurarine chloride containing *p*-chloro-*m*-cresol as a bacteriostatic agent it was found that this substance had an antagonistic action on the curarizing activity. This type of antagonism has been reported previously by Rothberger (1902), Mizuno (1933), and Coppée (1943), but has never been described for isolated mammalian tissues nor for pure *d*-tubocurarine chloride, and in view of the present-day practice of using phenolic substances as bacteriostatic agents it seemed desirable to investigate it further. Considerable confusion exists as to the identity of the so-called *p*-chloro-*m*-cresol ; the *British Pharmacopoeia* (1948a) records it as 6-chloro-3-hydroxytoluene, and (*B.P.*, 1948b) quotes Wien (1939) as having established its toxicity, whereas Wien used 4-chloro-3-hydroxytoluene which is stated to be *p*-chloro-*m*-cresol in the *Merck Index* (1940). The *p*-chloro-*m*-cresol we have used is 6-chloro-3-hydroxytoluene, m.p. 63–64° (the m.p. of the 6-chloro-compound is variously given as 52–66° whereas that for the 4-chloro-compound is 46°).

METHODS

Rat diaphragm-phrenic nerve preparations as described by Bülbring (1946) have been used throughout these experiments. The capacity of the bath was 100 c.c. A fresh preparation was used for each compound. Our Ringer-Locke, aerated with oxygen, contained 0.5 g. sodium bicarbonate/litre as opposed to the usual 0.15 g./litre. Square wave impulses of maximal strength and 0.34 or 6.7 msec. duration were applied to the nerve through a fluid electrode at the rate of 5 per minute and the contraction recorded isotonically.

RESULTS

From *p*-chloro-*m*-cresol we traced the antagonism through the three isomeric cresols to phenol. Toluene was too toxic to the preparation to give any satisfactory result. Next the antagonism was demonstrated in the dihydroxybenzenes, being most potent in catechol. Of the trihydroxybenzenes, phloroglucinol was the only one in which we found the action ; pyrogallol was too powerful a reducing agent and hydroxyhydroquinone was not available. Benzyl alcohol, salicylic acid, α- and β-naphthol, 2 :2′-dimethoxydiphenyl ether, veratrole, and 1 : 3-dimethoxybenzene were inactive. In addition to antagonizing *d*-tubocurarine, catechol and phenol reduced the action on a rat diaphragm of the triethiodide of 1 : 2 : 3-tri (β-diethylaminoethoxy) benzene (R.P. 3697), of the erythrina alkaloids, β-erythroidine, and dihydro-β-erythroidine, and of strychnine ethobromide the action of which is not reversed by neostigmine. They produced only a very slight antagonism of decamethonium iodide. The addition of catechol or phenol before or after tubocurarine did not affect the result.

When it became apparent that the relative antagonistic activities of a series of phenolic compounds had to be determined, a test of standardized design was decided upon. Essentially this was to find that dose of antagonist (AD66/33) which would reduce two-thirds paralysis, produced by *d*-tubocurarine alone, to one-third. In practice, this was found by measuring the paralysis in the presence of two doses of *d*-tubocurarine, the doses being selected so as to produce, by themselves, about 80 and 50 per cent paralysis respectively. Logarithmically spaced doses of antagonist were added to each dose of *d*-tubocurarine and the paralyses displayed by the mixtures were recorded. Since the relationship between log-dose *d*-tubocurarine and percentage paralysis is known to be linear in the absence of antagonizing substances (Fig. 1), at least between 20–80 per cent paralysis (Chou, 1947 ; Trevan, 1948) and

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since this linearity held within the experimental limits in the presence of phenols tested at three or more concentrations of *d*-tubocurarine, the graphical method of interpolation of results shown in Fig. 2 was employed. Here, degree of paralysis is plotted against dose of *d*-tubocurarine for each level of antagonist. By drawing an ordinate through the point on the upper control lines showing 66.7 per cent paralysis the percentage for each dose of antagonist corresponding to this degree of control may be read off. These figures may then be plotted against log-dose antagonist as in Fig. 3 and the AD66/33 determined directly.

The advantages of this design of test are that it is balanced about the 50 per cent paralysis point and that, by standardizing the effect and not the dose of *d*-tubocurarine (cf. Blaschko *et al.*, 1949), the variation between rat diaphragms is eliminated.

Statistical examination of the paralysis values obtained with varying doses of antagonists in the presence of the two-thirds paralysing dose of *d*-tubocurarine has shown that for each drug these are linearly related to log dose. A χ^2 test applied to the slopes of the regression lines for eight drugs gave a value of 13.47, which is not significant at the 5 per cent probability level. Thus a common slope of 30.93 (per cent per tenfold dose increment) could be given to each substance and its AD66/33 computed. Comparison of the values so obtained was then valid. These comparisons are recorded in Table I and include activities relative to phenol, both weight for weight and on the basis of molecular weights. The fiducial limits (p 95) of the relative activities are included, and show that, while

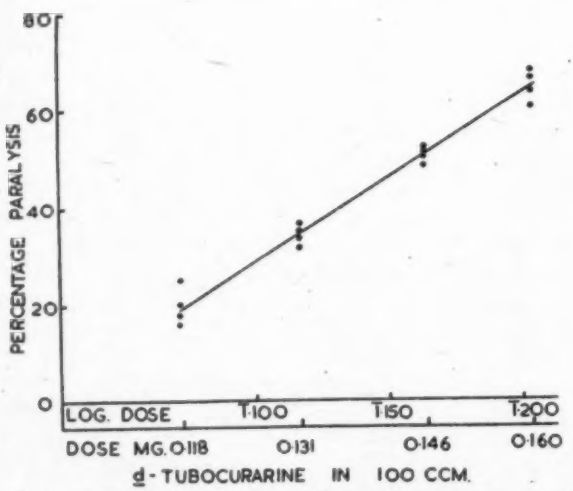

FIG. 1.—Dose-response-curve for *d*-tubocurarine on rat diaphragm showing linearity of regression. Doses (in mg.) are plotted logarithmically.

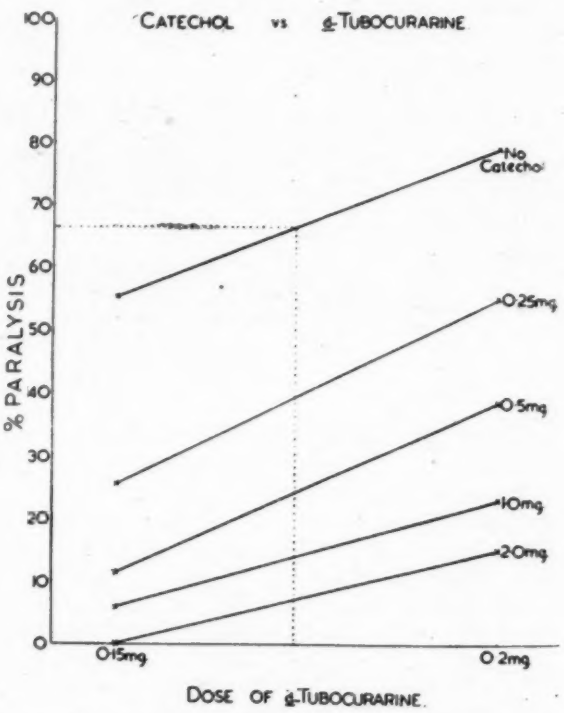

FIG. 2.—Reduction of paralysis of rat diaphragm by increasing doses of catechol added to two doses of *d*-tubocurarine. The vertical broken line is used to estimate the paralysis expected with each level of catechol in the presence of a two-thirds paralysing dose of *d*-tubocurarine.

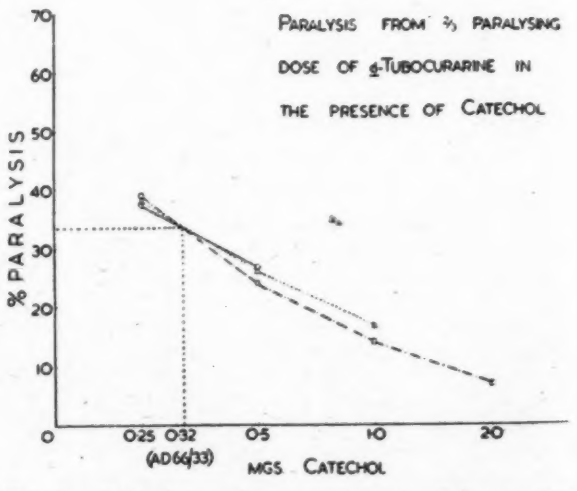

FIG. 3.—Data drawn from experiments similar to that illustrated in Fig. 2 employing 4, 3, and 2 doses of catechol. AD66/33 is the weight of catechol, read off the logarithmic scale, reducing the control two-thirds paralysis to one-third.

TABLE I

PHENOLIC ANTAGONISTS OF TUBOCURARINE CHLORIDE

Activity of antagonists estimated on rat diaphragm preparations and expressed as (1) AD66/33 or that amount of antagonist which reduces by half the activity of an amount of tubocurarine which by itself would have caused 66.7 per cent paralysis, (2) activity relative to phenol on a weight-for-weight basis, and (3) relative to phenol on a molar basis. In column (2) the p 95 limits are given in parentheses. Hydroquinone, guaiacol, and phloroglucinol were too weak to give an AD66/33.

Antagonist	(1) AD66/33 mg.	(2) Relative activity	(3) Relative molar activity
Catechol ...	0.30	5.0 (3.60–7.02)	5.9
p-Chloro-phenol	0.52	2.9 (1.89–4.56)	4.0
o-Cresol ...	0.62	2.5 (1.37–4.46)	2.8
o-Chloro-phenol	1.36	1.1 (0.78–1.61)	1.5
m-Cresol ...	1.37	1.1 (0.76–1.62)	1.3
Phenol	1.52	1.0	1.0
p-Cresol ...	1.64	0.9 (0.57–1.52)	1.1
Resorcinol ...	2.96	0.5 (0.35–0.75)	0.6
Hydroquinone	—	0.3	0.32
Guaiacol ...	—	0.1	0.13
Phloroglucinol	—	0.1	0.11

the actual relationships may not be too clearly defined, there is nevertheless a distinct fall of potency down the series. The results for hydroquinone, guaiacol, and phloroglucinol were similarly obtained by extrapolation of their graphs. The values given for these three cannot be regarded as more than an indication of their relative inactivity, however, as at high concentrations their paralysis-dose curves flattened out and with hydroquinone there was a complicating contracture of the diaphragm.

The results in Table I are essentially similar to those quoted by Coppée (1943). There does not appear to be any relationship between structure and action.

In order to investigate the absolute relationship between antagonist and *d*-tubocurarine, an alternative method of treating the information obtained from these tests was employed. The reduction in paralysis produced by the addition of antagonist to a given dose of tubocurarine may be regarded as equivalent to the removal of a portion of the tubocurarine. The mean slope of the paralysis/log dose *d*-tubocurarine regression line for the duration of the experiments was computed as being 252. If the paralysis produced by a known dose of *d*-tubocurarine be measured and its value plotted against log-dose, a line possessing the standard slope may be constructed. If, now, the paralysis be reduced by the addition of antagonist to the bath, the dose of tubocurarine which by itself would be expected to cause this smaller paralysis can be read off the graph. The difference between this dose and that in fact present may be regarded as the quantity of tubocurarine "neutralized" by the dose of antagonist. Each antagonist could then be represented by a curve relating its equivalence by weight with *d*-tubocurarine. The family of curves so obtained reflected the relative potencies already observed, in that they formed a series of parallel curves, parabolic in nature, lying in order one above another on the *d*-tubocurarine scale. The weights quoted above as AD66/33 correspond in this method to the weight of antagonist equivalent to 0.045 mg. *d*-tubocurarine, since this is the difference between the dose of tubocurarine required to produce two-thirds paralysis and that required to produce one-third paralysis.

While this method of plotting results seemed to indicate a constant activity of the different antagonists relative to one another, it was apparent that their relationship to *d*-tubocurarine was

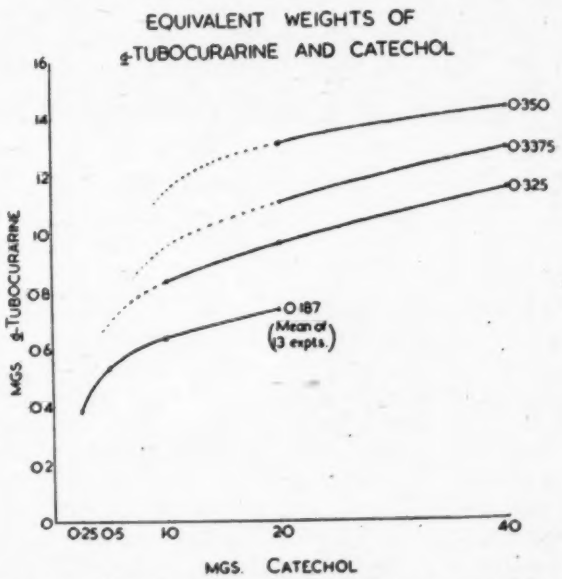

FIG. 4.—Neutralization of *d*-tubocurarine by catechol. The mean doses (mg.) of *d*-tubocurarine are indicated against the separate curves which show that the effectiveness of catechol increases as the *d*-tubocurarine concentration increases.

not stoichiometric. It appeared, moreover, that the antagonists became progressively less efficient neutralizing agents as their concentration increased. Indeed, it seemed possible that the curves were asymptotic, each substance having a limiting weight of tubocurarine which it could neutralize under our experimental conditions. However, closer examination of the accumulated data showed that the weight of tubocurarine neutralized by any one dose of antagonist increased as the actual dose of tubocurarine increased. An experiment to illustrate this point was conducted with catechol. Fig. 4 shows the mean curve for thirteen experiments in which the mean dose of *d*-tubocurarine was 0.187 mg., and three further lines obtained when catechol was added to increasing doses of tubocurarine. The latter three doses represented an excess of tubocurarine over the dose (approximately 0.275 mg.) required to produce 100 per cent paralysis.

However, when more and more excess *d*-tubocurarine is added a stage is eventually reached at which catechol will not antagonize the paralysis. This is reminiscent of the failure of neostigmine to antagonize large doses of *d*-tubocurarine (Fig. 5)

FIG. 5.—Rat diaphragm in 100 c.c. Ringer-Locke. Indirect maximal stimuli 6.7 msec. duration at 5 per min. "Prostigmin" even at high concentrations fails to antagonize a large dose of *d*-tubocurarine (5 μg. prostigmin normally reverses the action of 0.2 mg. *d*-tubocurarine.)

which Trevan (1948) has suggested may be due to *d*-tubocurarine acting at two sites, at only one of which it is reversible. Very large doses of catechol cannot be used to antagonize large excesses of *d*-tubocurarine because at high concentrations catechol has a depressant effect on the muscle fibres.

Further study of the nature of the action of catechol, as given below, showed its essential difference from the action of an anticholinesterase drug. It has been shown that a rat diaphragm, stimulated through the phrenic nerve with square wave impulses of short duration (0.34 msec.) and maximal intensity, responds with a twitch, whereas longer stimuli (6.7 msec.) produce tetanic responses (Mogey and Trevan, 1948a). Fig. 6 shows a diaphragm responding with greater and lesser contractions to long and short stimuli and also illustrates how the twitch response to short stimuli is altered to a repetitive response by eserine. This artificially induced repetitive response is abolished by minute doses of *d*-tubocurarine, doses so small that they do not affect the naturally occurring

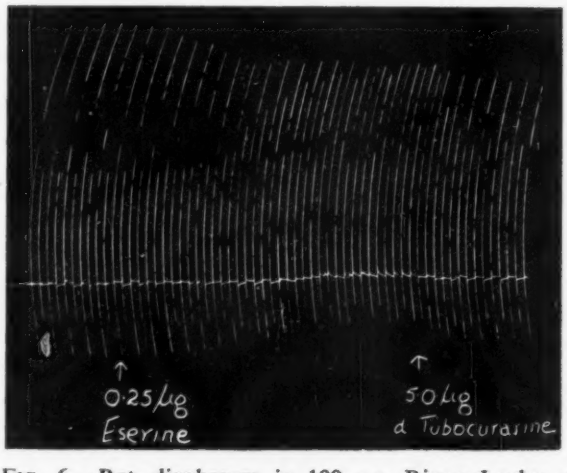

FIG. 6.—Rat diaphragm in 100 c.c. Ringer-Locke at 37° C. Alternate long (6.7 msec.) and short (0.34 msec.) stimuli of maximal intensity at 5 per min. to phrenic nerve. Eserine changes twitch to repetitive response and *d*-tubocurarine obliterates the artificially induced repetitive response at concentrations which leave unaffected the naturally occurring brief tetanus to long stimuli.

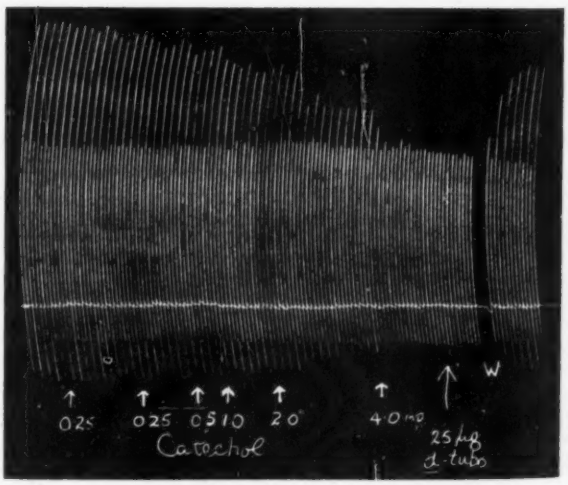

FIG. 7.—Conditions as in Fig. 6. Catechol fails to alter twitch responses while obliterating repetitive responses. *d*-Tubocurarine fails to restore repetitive responses, but they return after a wash.

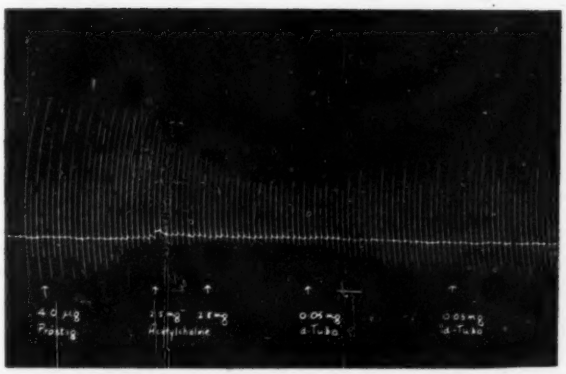

FIG. 8.—Conditions as in Figs. 6 and 7. *d*-Tubocurarine partially restores the response of the diaphragm after an acetylcholine-prostigmin paralysis.

repetitive responses to long stimuli. The ability to induce repetitive responses, common to many anticholinesterase agents, is not possessed by catechol. Fig. 7 illustrates the failure of catechol to increase the response of the diaphragm to stimuli of short duration at concentrations which increase the response during a tubocurarine-induced paralysis. Instead of inducing repetitive responses, catechol abolishes them as shown in the same figure, and, catechol having removed them, *d*-tubocurarine in low concentrations fails to restore them. This is in contrast to the action of tubocurarine on an acetylcholine paralysis (Fig. 8) (see also Bülbring, 1946—Fig. 12). Catechol failed to increase the response of the diaphragm to short stimuli of submaximal intensity and it did not improve the response of a fatigued preparation.

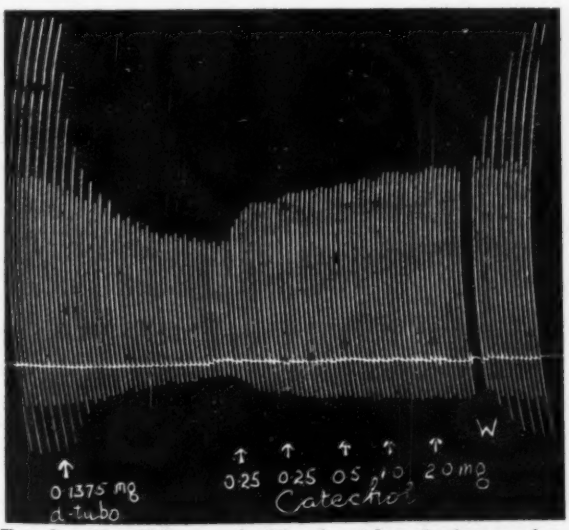

FIG. 9.—Conditions as in Fig. 6. After *d*-tubocurarine, catechol restores the twitch but fails to antagonize the action on the repetitive response to long stimuli.

The action of catechol on a tubocurarine-induced paralysis (Fig. 9) is different from that of the anticholinesterases—e.g., prostigmin as shown in Fig. 10. Its action is limited to restoration of the twitch response whereas prostigmin restores the repetitive response as well.

The anticholinesterase action of the hydroxybenzenes was examined manometrically by the method of Ammon (1933). None of those tested showed any marked inhibition of either true or pseudo cholinesterase. Results are presented in Table II.

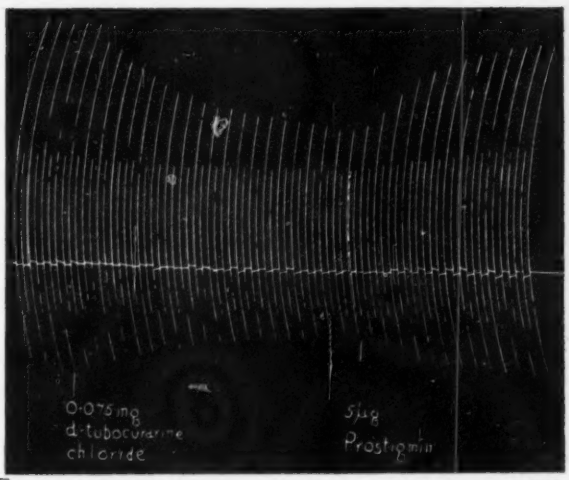

FIG. 10.—Conditions as in Fig. 6. Prostigmin, unlike catechol, restores the response to long stimuli after *d*-tubocurarine has reduced it.

TABLE II

ANTICHOLINESTERASE ACTIVITY (*in vitro*)

Concentration of inhibitor = $10^{-4}M$. Substrate for true (rat brain) cholinesterase: acetylcholine bromide. Substrate for pseudo (horse serum) cholinesterase: benzoylcholine iodide. (Each result is the mean of two determinations.)

Compound	Percentage inhibition of Rat brain enzyme	Horse serum enzyme
Phenol	4.0	1.0
Catechol	0.5	4.0
Resorcinol	8.0	0.0
Hydroquinone	9.0	0.0
Phloroglucinol	0.0	0.0
o-Cresol	1.0	1.0
m-Cresol	3.0	4.0
p-Cresol	5.0	5.0
p-Chloro-*m*-cresol ...	7.0	5.0
p-Chloro-phenol	3.0	7.0
o-Chloro-phenol	3.0	4.0
Guaiacol	0.0	0.0

The *p*H value of the Ringer-Locke, measured electrometrically, was not altered appreciably by either catechol or phenol at those concentrations which exhibited antagonism to *d*-tubocurarine.

Discussion

It is obvious that an explanation other than anticholinesterase activity must be sought for the antagonism between hydroxybenzenes and *d*-tubocurarine. Coppée (1943) has suggested that it is due to an " increase in the efficacy of the stimulus " by increasing the end plate potential (l'onde lente), but such an explanation fails to account for all the facts—e.g., catechol fails to restore repetitive responses to long stimuli after they have been removed by tubocurarine, it does not increase the response of the diaphragm to stimuli of short duration or of submaximal intensity (cf. adrenaline, Bülbring, 1946).

The antagonism cannot be due to an alteration of the action of *d*-tubocurarine by variations in H ion concentrations, for such changes are extremely slight and much too small to account for the marked antagonism. Similarly the action must reside in the hydroxybenzenes as such and not in the corresponding quinones unless it can be that the quinones, particularly *o*-benzoquinone, are highly active. Unfortunately none was available for testing.

We have not excluded a chemical combination between the antagonist and *d*-tubocurarine such as has been demonstrated for congo red and chlorazol fast pink by Kensler (1949), but such combination seems very improbable.

One other hypothesis presented itself but was subsequently proved to be false. It was based on the fact that the ratio of the potency of the dimethyl ether of *d*-tubocurarine to the potency of the phenolic alkaloid varies in different species (Mogey and Trevan, 1947 ; Collier *et al.*, 1948). For example, the dimethyl ether is three times as potent as the phenolic alkaloid in the rat, while it is only half as potent in the mouse. This suggested the possible importance of the hydroxyl groups in *d*-tubocurarine ; in some species (e.g., the rat) a methoxyl group might have a greater anchoring power than a hydroxyl, whereas in other species (e.g., the mouse) the reverse might be true. Perhaps the hydroxyl group of the hydroxybenzenes was attaching itself to the same receptor and so relaxing the grip of the tubocurarine. If this were so then we could have expected to predict the relative potencies of anisole, guaiacol, and veratrole as antagonists of *d*-tubocurarine and its dimethyl ether in the rat and mouse. Such expectations were not confirmed ; anisole was extremely toxic, causing contracture, veratrole was inactive against both *d*-tubocurarine and its dimethyl ether, and guaiacol gave results which could not be explained by this hypothesis. Catechol and guaiacol versus *d*-tubocurarine and its dimethyl ether on rat and mouse diaphragms are compared in Table III.

TABLE III

Catechol and guaiacol as antagonists of *d*-tubocurarine and its dimethyl ether on rat and mouse diaphragms.

A. RAT DIAPHRAGM

Antagonist	*d*-Tubocurarine chloride 0.2 mg.		*d*-Tubocurarine dimethylether 0.07 mg.	
	Dose of antagonist	% Paralysis	Dose of antagonist	% Paralysis
Catechol	None	75	None	79
	0.25 mg.	52	0.25 mg.	70
	0.5 mg.	36	0.5 mg.	64
	1.0 mg.	24	1.0 mg.	56
Guaiacol	None	83	None	75
	2.0 mg.	77	2.0 mg.	73
	4.0 mg.	75	4.0 mg.	73
	8.0 mg.	73	8.0 mg.	73

B. MOUSE DIAPHRAGM

Antagonist	*d*-Tubocurarine chloride 0.375 mg.		*d*-Tubocurarine dimethylether 0.7 mg.	
	Dose of antagonist	% Paralysis	Dose of antagonist	% Paralysis
Catechol	None	79	None	83
	0.25 mg.	26	0.25 mg.	12
	0.5 mg.	8	0.5 mg.	0
Guaiacol	None	91	None	89
	2.0 mg.	66	2.0 mg.	65
	4.0 mg.	54	4.0 mg.	51
	8.0 mg.	50	8.0 mg.	40

The mechanism of the antagonism is so far unexplained: it is possible that the solution lies in the almost universal attraction between phenols and proteins, thus causing steric hindrance.

Summary

1. A series of hydroxy-derivatives of benzene has been shown to antagonize the action of *d*-tubocurarine on the rat diaphragm.

2. The relative potencies of the members of the series have been expressed as the concentration of antagonist which reduces by half the effect of a concentration of *d*-tubocurarine which alone would cause two-thirds paralysis.

3. The antagonism has been shown not to be due to cholinesterase inhibition or alteration of *p*H. It is suggested that it is not due to a chemical combination of antagonist with *d*-tubocurarine, but the true mechanism has not been elucidated.

We are indebted to and are pleased to thank Dr. J. W. Trevan and Dr. A. C. White for their interest and advice, and Messrs. F. Huggins, D. J. Tulett, and G. Downs for technical assistance. We also wish to acknowledge the kind co-operation of Dr. F. C. Copp in supplying us with some of the compounds tested.

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PERSONAL ERROR IN PENICILLIN ASSAY

BY

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In many forms of biological testing the intrinsic errors of the methods used are so great as to render insignificant any manipulative errors on the part of the assayers. Some assays, however, have such small inherent errors as to make the personal factor of considerable importance. This paper will consider the problem as met with in the course of four years' routine penicillin estimations.

Method of Assay

The test employed was that described by Pope and Stevens (1945–6). Standard penicillin was dissolved accurately to 5 u./ml. in phosphate buffer *p*H 6.0; 2.0 ml. of this solution were pipetted into 20 ml. of test broth and a range of six volumes delivered from the broth dilution into six tubes, each containing 20 ml. of broth. Three series of six standard tubes were set up for every thirty unknowns.

A typical series of volumes would be:

1.3	1.2	1.1	1.0	0.9	0.8
1.25	1.15	1.05	0.95	0.85	0.75
1.2	1.1	1.0	0.9	0.8	0.7 ml.

The procedure for unknown samples was to dilute each sample in buffer to approximately 5 u./ml., further dilute this 1/11 in broth, and deliver volumes of this dilution into a set of six tubes. The range of volumes employed may of course be varied. In addition to the 10 per cent ranges illustrated above (the percentage indicates the average dilution increment between adjacent tubes) we have commonly used:

20 per cent range	2.0	1.6	1.3	1.1	0.9	0.7 ml.
30 per cent range	2.0	1.5	1.1	0.85	0.65	0.5 ml.

and, less often:

90 per cent range	6.0	2.0	1.4	0.7	0.4	0.2 ml.

After addition of the penicillin to the test broth the latter was inoculated from a suspension of *Staph. aureus* (Oxford strain). Usually all the day's tests were set up with penicillin before beginning inoculation, although this order was reversed for a few months only (see below). Tests were incubated overnight at 37° C. in a controlled temperature room, and were then read visually by moderate indirect illumination. The end-point was taken as the minimum trace of growth detectable by eye, and the volume permitting this amount of growth was estimated to 0.05 ml. and recorded as "end-point volume." With practice it was possible to recognize degrees of growth corresponding to 5, 10, and 15 per cent less penicillin than that producing an end-point. Thus in a range of tubes with a dilution factor of 1.2 (20 per cent range) it was possible to place the end-point to within 5 per cent (approx. 0.05 ml.) should it fall between two adjacent tubes. The computation of results was completely covered by a system of tables which allowed for standard and unknown end-point volumes, and preliminary buffer dilution of the unknown.

Results

The sources of personal error in the technique were in the preparation of dilutions, delivery of volumes, and in the reading of the tests. In skilled hands and with the 10 per cent ranges, the standard deviation of a single test has often been of the order ±3–4 per cent. Such proficiency could only be obtained after much experience in assay, however, and even then the frame of mind of the worker was of great importance if maximum accuracy were to be achieved.

The problem which confronted this laboratory at the time of its inception in November, 1944, was one of training half a dozen assistants in the technique of penicillin assay. Only one had previous laboratory experience (L.B., male); the rest were girls whose ages ranged from 16 to 18, of whom all except one had matriculated and were fresh from school. After only one week of general training, including a few practice assays, it became imperative that some routine tests should be undertaken, although it was realized that these could not be expected to be of great accuracy. The demand for assays at that time was steadily mounting, and in order to make the assayers "accuracy-conscious" some simple form of "scoring" their work was sought. The method adopted was to test each sample submitted for assay four to eight times, the arithmetical mean of the results of these

tests being issued as the final answer. Each result was then expressed as a percentage of the mean, and these percentages plotted for the assayer responsible. When the number of tests performed by a worker exceeded forty in one week, only that number taken at random was treated in this way from the week's work. Fig. 1 reproduces this chart over a period of five weeks ; each chart was commenced during the assayer's first week. The improvement in the later weeks is very marked for some of the workers, all of whom entered into the competitive spirit which this comparison so easily fostered. However, it did not seem desirable to continue the charts after the five-week period, as the workers by then had more or less reached their maximum accuracy.

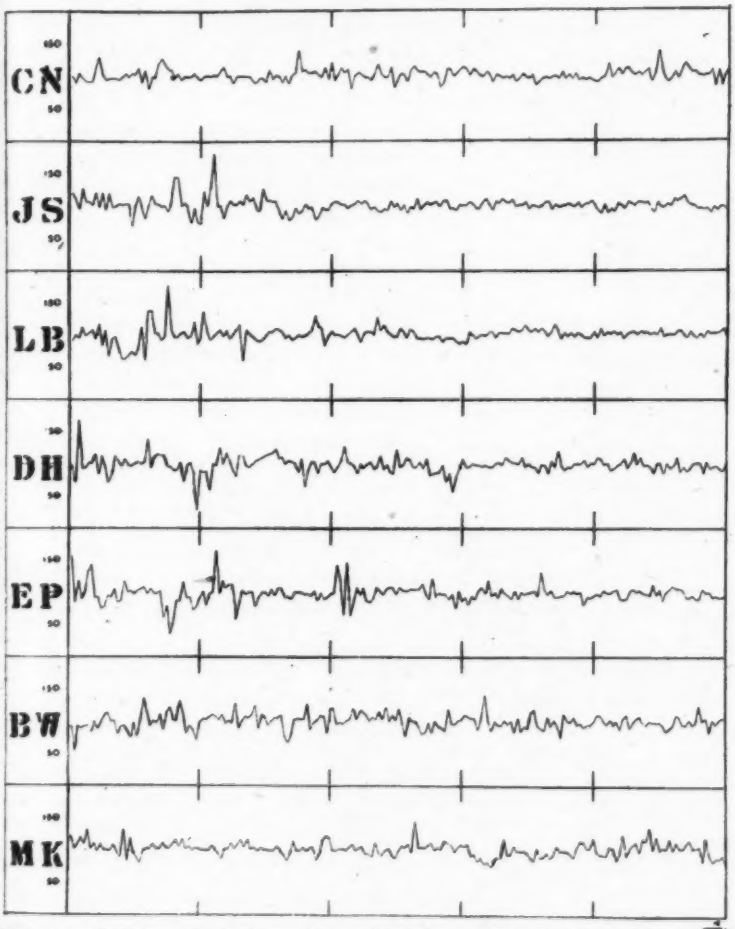

FIG. 1.—Worker deviation chart. Results obtained by each worker plotted as percentages of the means for all workers. Forty results at random taken from each week's tests for each assayer. The horizontal scale represents weeks. Charting commenced for each worker during the first week's work.

The percentages obtained for the preparation of the charts were also used to calculate the variance each week for each worker, and on the basis of the previous week's variances each assayer was assigned a weight in inverse proportion to his or her variance. For simplicity these weights were reduced to simple digits. The computation of the mean for each sample was then made by appropriately weighting each worker's values. This appeared to be particularly valuable when new staff were engaged to perform assays, as only one week's practice testing was required before they could be assigned a weight, and from then on they could enter into the routine assays without fear of their inexperience unduly influencing the results issued from the department. Further experience taught us, however, that week-to-week fluctuations in individual variance were unaccountably high. Consequently it was not valid to assess one week's work in terms of the previous week's variance, and the system of weighting was discontinued.

A study of the mean variance for all workers each week over the period, November, 1944, to March, 1945, revealed some interesting trends (Fig. 2). Initially, the mean variance was very high (S.D. for single tests ± 12.5 per cent). On the third week, however, this fell, and by the fourth week had reached quite a satisfactory level (S.D. ± 8 per cent). The value remained fairly steady for the next three weeks, but rose again to an alarming extent during the eighth week, only to fall during the succeeding weeks to an even lower figure than previously obtained. The point of interest is that the eighth week was in fact the three working days after Christmas. We appeared, therefore, to be recording the after-effects of festivities which, for five out of the seven staff, fell after only seven weeks' employment. Some of the workers had displayed greater excitement than the others about this time, and these were the ones whose testing demonstrated the greater variation. No such general rise in variance was observed around Christmas, 1945, by

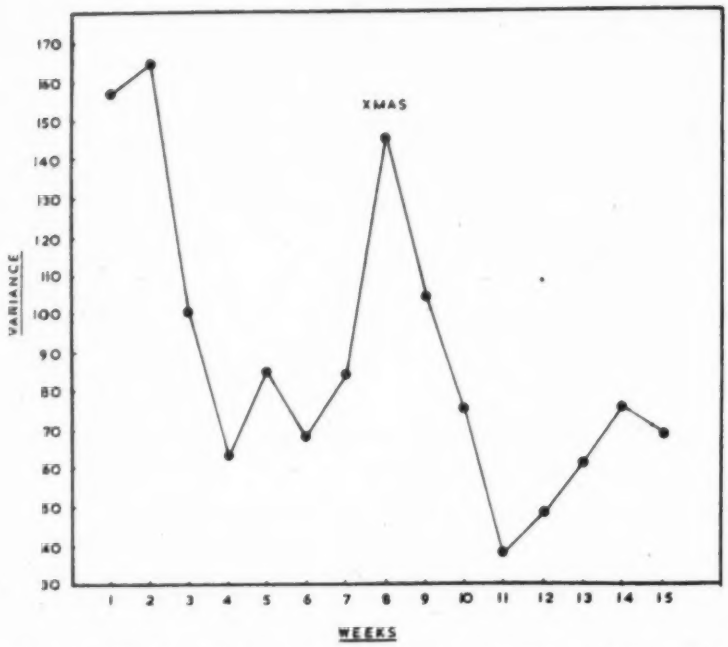

FIG. 2.—Weekly variance; mean values for seven workers. Each estimate plotted is associated with about 200 degrees of freedom.

which time the staff had naturally "settled down" to their employment.

From the eleventh week onwards some deterioration of the accuracy of our tests was noted. This period corresponded with the introduction of samples from the production unit; previously we had been assaying samples derived from small-scale laboratory experiments. The effect of this change was twofold: First, it was necessary to obtain a final answer for the production samples as quickly as possible. Second, whereas the laboratory experiments were conducted with great precision, on accurately assayed material, and the samples submitted for assay could very often have their potency predicted to some extent, the penicillin content of the samples from the large-scale plant was, for some time, quite unpredictable. The effect was to oblige us to test the plant samples on the wider ranges, 20, 30, and even 90 per cent steps being used. The relative accuracies of these ranges will be dealt with later, but it will be clear that the overall effect at the outset was to raise the general variance.

After fourteen weeks' work in the department it became too laborious to calculate individual variances each week. Instead the general variance was obtained by taking a hundred results at random from the week's work, each result being expressed as a percentage of the mean value for the sample from which it was obtained, and the variance of these percentages was calculated. The value so obtained was subject to a bias, particularly when only three or four results were used to obtain the mean value for a sample. Nevertheless, this statistical device seemed suitable as a general guide to the current standard of testing. When new staff came into the laboratory they were allowed to spend their first week in general training with the apparatus used, and thereafter were put on to the testing of samples which had already been assayed by the more competent workers. Their results on these samples were expressed as percentages of the values already assigned and the variance of these percentages determined. Usually after two to three weeks of such "duplicate" testing they were considered reliable enough to enter the routine of the laboratory. The weekly variances for six new arrivals are listed in Table I together with the general mean variance for the established staff over the first five weeks of their work. Fig. 3 shows the mean variance for thirteen assayers for each of their first five weeks of testing. It demonstrates very clearly that accurate testing could not be expected during the first fortnight, after which the variance drops rapidly until, at four to five weeks, the curve markedly flattens out, and, at five weeks, reaches the value of 80 for the variance, which was exactly the average value for the

TABLE I

WEEKLY VARIANCE OF TRAINEES

Each estimate is derived from 40 results drawn from the week's work. The general mean (for the established staff) is the mean of the 5 variances for the respective weeks, each derived from 100 results.

Worker	Week of testing					General mean for weeks 1–5
	1st	2nd	3rd	4th	5th	
RK ...	461	540	327	149	182	103
SG ...	388	396	91	120	—	85
NB ...	228	73	87	58	84	104
KD ...	467	199	111	101	89	111
SR ...	184	179	120	98	48	115
JP ...	110	66	79	117	77	103

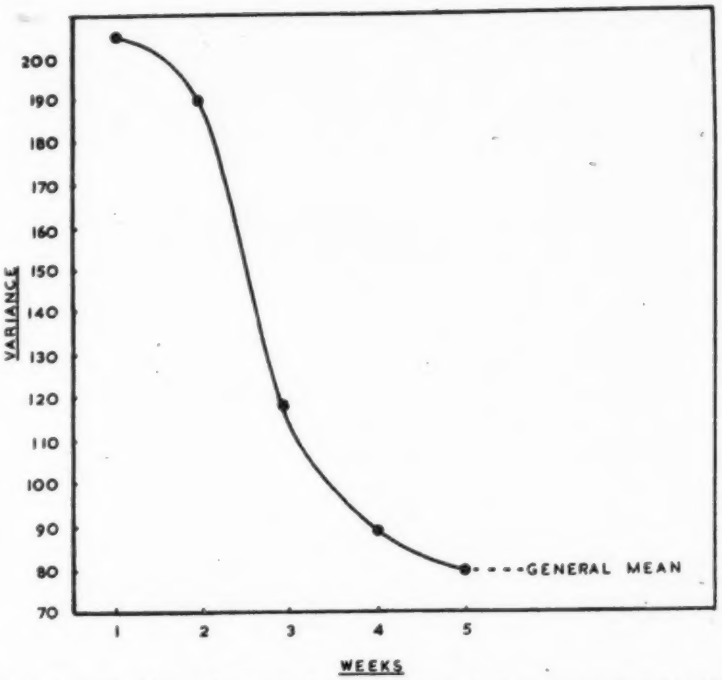

FIG. 3.—Weekly variance during training period; mean values for 13 workers. The general mean is that for the established staff for the 5th week. Each estimate plotted is associated with about 500 degrees of freedom.

general mean variance corresponding to the fifth week.

It should be noted at this stage that the procedure in the laboratory was to record details of samples received in a log book, and to label the samples with a sample number. Test sheets were then prepared showing the sample numbers, the buffer dilution required for each, and the volumes in ml. to be delivered to the test broth. One worker would then prepare the buffer dilutions of the samples and another, the "tester," would be responsible for delivering the test volumes. The latter worker would also be responsible for reading her own tests after overnight incubation. Thus the variance in the results would be shared between the worker preparing the dilutions and the "tester." It has always been felt that the contribution of dilution preparation to assay variance was relatively insignificant, a fact more or less borne out by consideration of the effect of the range used on the variance of results; this effect is illustrated in Fig. 4, where the variance associated with different levels of titre (u./ml.) is plotted. In general the samples of lower titre were tested on the wider ranges and these tests were inevitably liable to greater variance. The titre range 5–100 u./ml. is given the variance for dried products which were dissolved at concentrations falling within these limits of potency. These solutions were tested on the 20 per cent range. The unexpectedly low figure for the 90 per cent range was brought about by the fact that some of the results taken for its computation were derived from closer range tests, it not being possible from the records available to say exactly which range had been used. The variances of 51 and 85 for the two highest titre groups reflect an increase due to the necessity for serial buffer dilution in the preparation of samples in the higher of the two groups. The increase, however, cannot be directly assessed as the result of two buffer dilutions compared with one in the lower groups because the physical properties of the concentrated penicillin solutions make their measurements far more difficult.

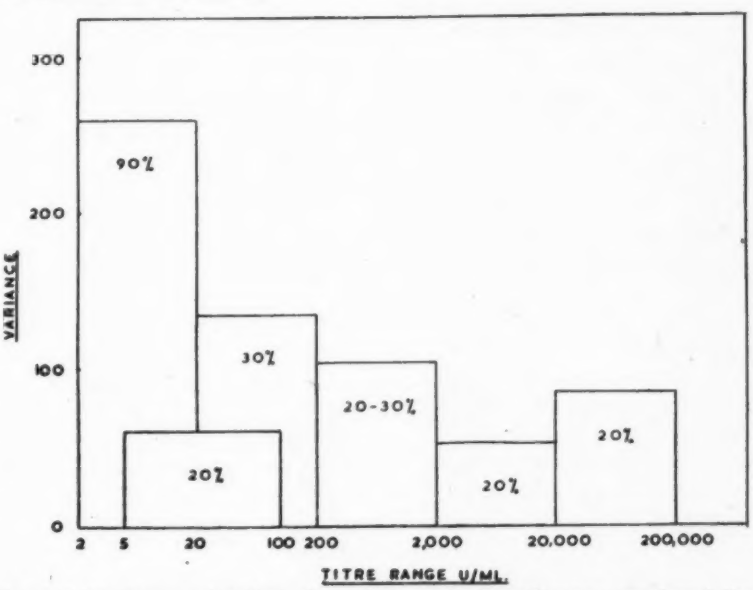

FIG. 4.—Variance related to titre of the sample and range employed for testing. Each variance is derived from 80 observations.

The general variance each week was particularly of use when, for any reason, a change in technique became necessary. It was found, for example, in October, 1945, that the variance had risen to an undesirably high level, and the cause of this rise was not known. By an internal reorganization of the department it was arranged that each tester should perform only half the number of tests as hitherto, since it was believed that the prevalent inaccuracy might be due to an excess of work. This arrangement did, in fact, reduce the S.D. for a single test from ±14.2 to ±11.8 per cent, which figure was still considered unsatisfactory. The next step was to introduce delivery pipettes in place of blow-out pipettes for the setting up of tests. The immediate effect of this was to raise the S.D. to ±13.7 per cent, but after a week's use of the new type of pipette the figure fell again to ±11.8 per cent for the second week and ±12.2 per cent for the third week. It appeared that the high variance first obtained with the new pipettes was due to unfamiliarity with their calibration, but that after some practice no advantage was gained by their use. Finally it was decided to discontinue the practice, which we had adopted some months previously, of inoculating the test broth with a suspension of *Staph. aureus* before introduction of the penicillin volumes. We found that by inoculating the broth after penicillin had been added the S.D. immediately dropped to ±9.0 per cent; this was considered satisfactory for the type of assays in hand and no further modifications seemed desirable. It may be interesting to record that when, some four months later, considerable impending reductions in staff were announced, the effect was to raise the S.D. from about ±9.7 to ±15.0 per cent, but that after one week it again fell to below ±10.0 per cent. This again reflected the emotional interference with the accuracy of the work.

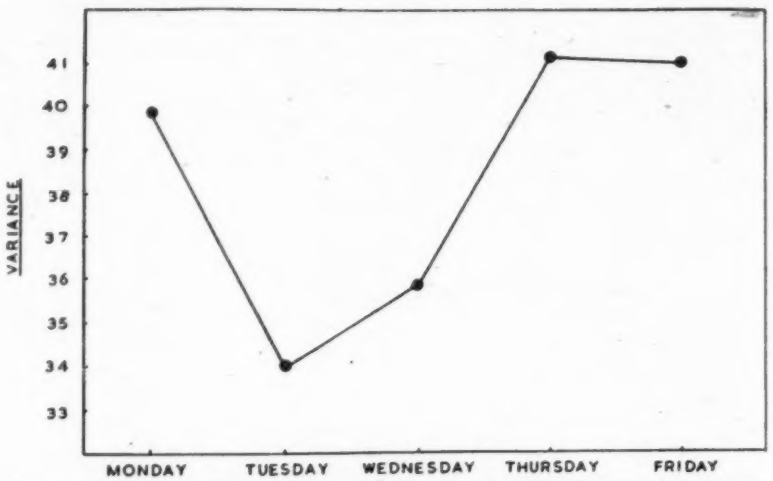

FIG. 5.—Daily variance; mean results for 3 girls; 280 observations per day

In view of the personal factor involved in this form of assay I was interested to find out whether the day of the week on which tests were carried out might influence the accuracy of testing. Variances were, therefore, calculated on results obtained from tests performed on each day of the week. At first a hundred results were examined for each day taken at random over a 12-week period. The variances so obtained did in fact show a tendency to reach a minimum at the middle of the week and to average at more or less the same high level both at the beginning and at the end of the week. Over the period involved there were seven assayers who averaged seventy tests each day. A similar analysis was made on results eighteen months later, when over a period of ten weeks three girls averaged forty-four tests each day. For each day of the week two hundred and eighty observations were made; the variances obtained are plotted in Fig. 5. Again the middle of the week would seem optimal from the point of view of accuracy, although here Tuesday is even better than Wednesday. Since each sample was always assayed on two successive days, the variances attributed to one day would be influenced to some extent by the preceding and following days.

Since the mid-week improvement in accuracy shown above appeared to be real it was natural to inquire whether any similar relationship held between variance and the time at which tests were carried out. It was possible to obtain assessments of the variance figure corresponding to tests set up at varying times under three different sets of conditions. First, during the peak period of penicillin assay, when testing took up three and a half hours in the afternoon, and approximately one and a half hours in the morning devoted to reading the tests. The setting up of tests during this period was at the rate of approximately one test every two minutes. Later, when owing to changed circumstances penicillin testing occupied only one hour or less of the day, it was possible to obtain variances at short time intervals: (*a*) When the total number of tests performed on one day was not greater than thirty-two, and (*b*) when the total

number was greater than thirty-two, and averaged about sixty. These tests were all performed at the rate of about one per minute, and were set up on a 10 per cent range, while the first series were set up on a 20 or 30 per cent range. Fig. 6 shows the variance plotted against the time in minutes after commencement of testing. It will be seen that the variance always rose in the course of testing, and

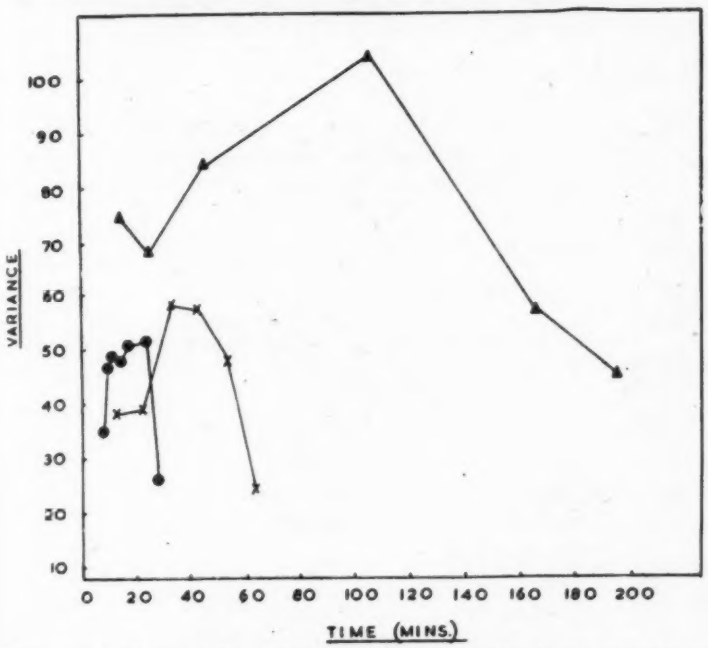

FIG. 6.—Variance at times during testing. The time is measured from the commencement of assays for the day. The three curves relate to different periods and represent the total penicillin assays carried out per day within each period. Each value associated with about 45 degrees of freedom.

fell again towards its completion. This would imply a subconscious timing by the tester, which would give the beginning and end of her work a greater significance than the middle, and would entail greater concentration to the measurements involved. Such an argument would apply equally to the setting up and to the reading of tests. Since occasional check readings have usually agreed closely with the originals, it would appear that the chief errors must enter during the setting up of the tests. It is probable that a form of rhythmical pipetting might commence once the testing had been started ; this would particularly affect the two shorter series of assays since the volume ranges in use at that time were invariably the same for all samples. Since the final increase in accuracy did not appear to be affected by the duration of testing, it would seem that no question of fatigue was involved. Moreover the mean variance for thirty tests (45.4) did not differ significantly from that for sixty tests (43.9). The mean variance for the longest series (71.1) was, without a doubt, higher by reason of the wider ranges used.

DISCUSSION

It must be admitted that many of the effects described above are partially confounded ; for example, the error of one worker's testing may be assessed only by comparison with the results of other testers, and is, therefore, subject to error on that account. Unfortunately, too, the analysis of errors between days, and within days, was only completed after the laboratory had ceased to assay penicillin samples. Consequently it was not possible to impose any design upon the assay procedure which would have yielded results free from confounding. Nevertheless the paucity of literature on the subject of personal error in routine occupations has prompted the author to present his findings.

Much information has been published on tests designed to detect accident-proneness among varied groups of workers. Of this, some experiments carried out at the request of the Flying Personnel Research Committee appear to be most pertinent to the present problem. Outlined by Bartlett (1943) the work is fully reviewed by Davis (1948) in an Air Ministry publication. The purpose of the experiments was to enumerate the deviations from an ideal pattern of behaviour of pilots subjected to tests in an experimental cockpit, and to relate their findings to the subsequent accident history of individual pilots. In the first instance, therefore, it was the variance of behaviour that was being assessed. These authors found that the number of deviations exceeding arbitrary limits within fixed time periods rose from the beginning of the test, reached a maximum at about the middle, and fell off towards the end. If the number of deviations they observed be taken to be a function of the variance, then such observations would parallel the results shown in Fig. 6.

Whether the psychological interpretations of their results put forward by Bartlett and Davis could be applied to the simple routine of penicillin assay will not be discussed here at length. Nevertheless, it seems likely that there is a mental

process common to these extremely different types of behaviour, since in each the disorganization of skill appears to be independent of physical fatigue.

It has already been stated that the variance for individual workers may change considerably from week to week and from day to day. The magnitude of this "variance of variance" is probably a better guide to the efficiency of the worker than the mean variance over a long period. The latter may, in fact, fail to reveal any significant differences between workers. Thus it has been observed that a worker with a mean variance lower than that of her colleagues had a variance of variance (variance measured weekly over a twelve-week period) considerably greater. This type of worker has usually been observed to be subject to "moods," or periods of depression, between which their work is excellent, but during which it is most unreliable. For none of the female workers has a monthly cycle been observed in individual variance, however, but sickness, possibly of nervous origin, has been associated with an increase in errors. One girl subject to migrainous headaches was quite incapable of reading her tests during attacks.

Summary

A method is described for the estimation of the variance of results obtained in the course of routine penicillin assays. In particular this is applied to investigate the effect of personal error on the results of the tests. Its use in the control of laboratory technique is also indicated.

I would like to thank Miss Dawn Hayles and Miss Joyce Peacock for their assistance in many of the computations involved in this study, and to acknowledge the encouragement of my colleagues on the staff of the Wellcome Penicillin Unit in whose service the assays were carried out.

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THE ACTION OF *NOR*ADRENALINE

BY

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Several investigations of the properties of *nor*-adrenaline (arterenol) have been published since the original observations of Barger and Dale (1910). As a list of these is given in the recent paper by Luduena, Ananenko, Siegmund, and Miller (1949) no attempt will be made to summarize previous work here, though the observations of others will be discussed in describing our own experiments. These have been carried out in order to investigate more closely the vascular action of *nor*-adrenaline, especially its vasodilator properties, to study further the effect of denervation upon its action, and also the variation in the relative potency of adrenaline and *nor*adrenaline in different organs. We have had at our disposal synthetic *l*-adrenaline and also a sample of *dl-nor*adrenaline kindly given to us by Dr. M. L. Tainter.

The cardiovascular system

Although it has long been known that agents like ergotoxine which reverse the pressor action of adrenaline fail to reverse that of *nor*adrenaline (Barger and Dale, 1910), hitherto there has been no demonstration in an animal corresponding to the results of Goldenberg, Pines, Baldwin, Greene, and Roh (1948) in man. These authors found that when the two substances were infused intravenously, changes occurred which enabled them to calculate that adrenaline caused a decrease of the general peripheral resistance, whereas *nor*-adrenaline caused an increase. Barcroft and Konzett (1949) infused the two substances intravenously in man, and found that, whereas *nor*-adrenaline caused a rise in systolic and diastolic pressure with a slowing of the heart rate, adrenaline caused a smaller rise in the systolic pressure and a fall in the diastolic pressure with a quickening of the heart rate.

Action on blood pressure.—The depressor action of adrenaline described by Moore and Purinton (1900) can be most easily observed in the cat when the animal is anaesthetized with ether and the vagi are cut. Under these conditions the blood pressure is usually high. When adrenaline is infused into a vein at a slow rate, there is a fall of blood pressure. When 5 μg. *l*-adrenaline were infused during 2 min., the fall recorded in Fig. 1*a* was produced. When 10 μg. *dl-nor*-adrenaline were infused during 2 min., there was a slight rise of blood pressure. The effects were then repeated in (*c*) and (*d*).

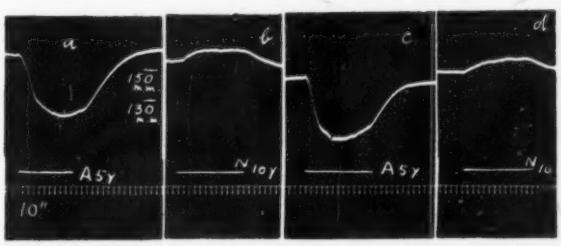

FIG. 1.—Cat blood pressure under ether anaesthesia after section of both vagi. (*a*) Shows depressor action of adrenaline when 5 μg. were slowly infused during 2 min. (*b*) Shows slight pressor action of *dl-nor*adrenaline when 10 μg. were similarly infused. (*c*) is a repetition of (*a*), and (*d*) is a repetition of (*b*).

The volume of one hindleg was also recorded in this experiment by using a plethysmograph, but except for an increase in volume pulse during adrenaline infusion no change was observed. Dale and Richards (1918) showed that if the hindleg was denervated by section of the sciatic nerve seven days previously the infusion of adrenaline caused a dilatation of the hindleg. By section of the nerves the reflex change in vascular tone resulting from a change in blood pressure was excluded. Three cats were therefore prepared by sciatic section and the final experiment in one of these is illustrated in Fig. 2. The infusion of 10 μg. *l*-adrenaline during 30 sec. produced a fall of blood pressure and dilatation of the denervated leg. The infusion of 20 μg. *dl-nor*adrenaline during 30 sec. produced a rise of blood pressure and constriction of the leg. The results, which were repeated several

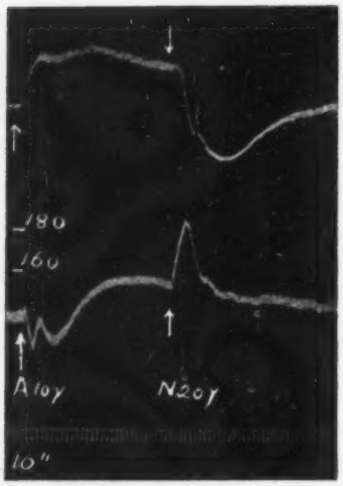

FIG. 2.—Cat under ether; vagi cut. L. sciatic nerve cut 8 days previously. Upper record is volume of the left hindleg and lower record is blood pressure. At the first arrow 10 μg. adrenaline was infused in 30 sec., and at the second arrow 20 μg. *dl-nor*adrenaline was infused in 30 sec. The first injection caused dilatation of the limb volume; the second caused constriction.

times in each of the cats, show that *l*-adrenaline caused peripheral dilatation but that *dl-nor*adrenaline caused peripheral constriction, the changes in the blood pressure being due to these effects.

Rabbit ear vessels.—In the rabbit ear vessels it is possible to study not only the vasoconstrictor action of adrenaline but also its vasodilator action which Gowdey (1948) showed was exerted during perfusion with Locke's solution containing 2-benzylimidazoline in a concentration 0.2×10^{-3}. We have therefore used the rabbit ear to compare the action of *nor*adrenaline with that of adrenaline in the presence of this reversing agent. The constrictor action of *nor*adrenaline on the vessels of the rabbit ear has been examined by Luduena *et al.* (1949) and also by Gaddum, Peart, and Vogt (1949). The former found that the ratio of equiactive amounts of *l-nor*adrenaline and *l*-adrenaline was 1.5–2.5; the latter found the ratio was 1.0–3.0. We made 21 comparisons and found the ratio was 1.0–4.0. All these findings agree in showing that as a rule *nor*adrenaline is a less potent constrictor than adrenaline for rabbit ear vessels, though the variation is from 25 to 100 per cent. In one experiment in which the dog hindleg was perfused with blood by a pump, we found that 4 μg. adrenaline and 8 μg. *dl-nor*adrenaline were equally active.

We found that perfusion of the rabbit ear with 2-benzylimidazoline converted the vasoconstrictor

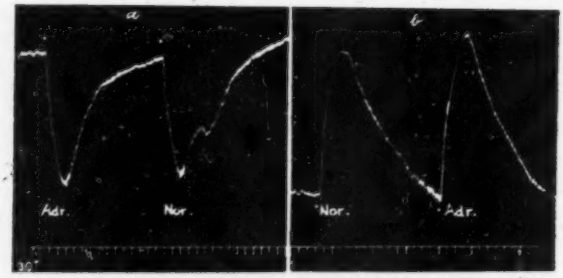

FIG. 3.—Rabbit ear vessels perfused with Locke's solution. Outflow recorded with Stephenson's recorder. (*a*) Shows equal constrictor effects of 0.04 μg. adrenaline and of 0.25 μg. *dl-nor*adrenaline. (*b*) Shows effects obtained during perfusion with Locke containing 0.2×10^{-3} benzylimidazoline. Equal dilator effects were exerted by 0.5 μg. *dl-nor*adrenaline and 0.15 μg. adrenaline.

action of *nor*adrenaline into a vasodilator action; this conversion is shown in Fig. 3, in which (*b*) shows that 0.16 μg. *l*-adrenaline caused a dilatation similar to that caused by 0.5 μg. *dl-nor*adrenaline. The vasodilator ratio of *l-nor*adrenaline to *l*-adrenaline was therefore about 1.5. The vasoconstrictor ratio in Fig. 3*a* was 3.0. The results of a series of observations are given in Table I. These indicated that the relative dilator action is similar to the relative constrictor action in the majority of ears; only in occasional ears like No. 5 in Table I is the dilator action of *nor*adrenaline very feeble. The conditions in the

TABLE I

COMPARISON OF DILATOR ACTION OF ADRENALINE WITH THAT OF *nor*ADRENALINE IN RABBIT EAR VESSELS DURING PERFUSION OF 2-BENZYLIMIDAZOLINE

Exp.	Equidilator doses		Ratio *l-nor*adrenaline/ *l*-adrenaline
	l-adrenaline μg.	*dl-nor*adrenaline μg.	
1	0.16	0.48	1.5
2	0.1	0.2	1.0
3	0.3	1.0	1.67
4	0.2	0.4	1.0
5	0.1	10.0	50.0
6	1.0	2.0	1.0

rabbit ear appear to be the opposite of the conditions in the cat, judged by the depressor action, seen after giving ergotoxine or ergotamine. In the majority of cats the depressor action of *nor*adrenaline is feeble or absent unless large doses are given (West, 1949), but in occasional cats it is just as great as that of adrenaline, as is shown in Fig. 4.

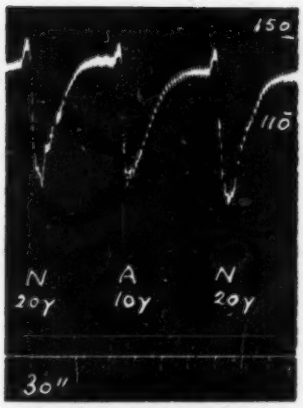

FIG. 4.—Cat, spinal preparation, eviscerated. Blood pressure record. Ergotoxine ethane sulphonate was injected in order to reverse action of adrenaline. 20 μg. *dl-nor*adrenaline was injected causing a depression similar to that caused by 10 μg. adrenaline. This result was exceptional.

Intestinal vessels.—Plethysmograph experiments were carried out on loops of the intestine of the cat under chloralose anaesthesia using the method described by Bülbring and Burn (1936). It was found that the injection of a small dose of *dl-nor*-adrenaline caused dilatation of the intestinal loop just as did the injection of a similar dose of adrenaline, as shown in Fig. 5. *Nor*adrenaline caused some rise of blood pressure, though this passed off as the dilatation came on. However, to exclude the blood pressure rise as a cause of the dilatation, some experiments were done in which the blood pressure was kept constant by attaching the carotid cannula to a large vessel containing Ringer's solution at constant pressure. The same dilatation of the intestinal loop was observed when *nor*adrenaline was injected, although the blood pressure did not change.

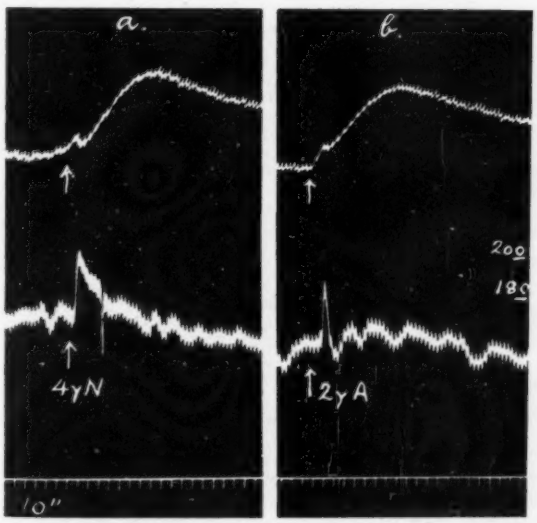

FIG. 5.—Cat, chloralose. Upper record shows volume of intestinal loop; lower record is blood pressure; (*a*) shows dilatation of intestinal vessels when 4 μg. *dl-nor*adrenaline was injected; (*b*) shows similar dilatation when 2 μg. adrenaline was injected.

Action on the heart.—Since *nor*adrenaline lacks the vasodilator action of adrenaline in the muscle vessels, it was of interest to discover the effect of *nor*adrenaline on the coronary vessels. Observations were first made on the coronary flow, the rate, and the amplitude of the cat heart perfused with Ringer-Locke by Langendorff's method. Fig. 6 shows the changes in coronary flow with Stephenson's recorder (1948). The effects of the two substances in the doses given were almost identical on the amplitude and coronary flow, though *dl-nor*adrenaline had less effect on the rate, increasing it by 54 beats per min., while adrenaline

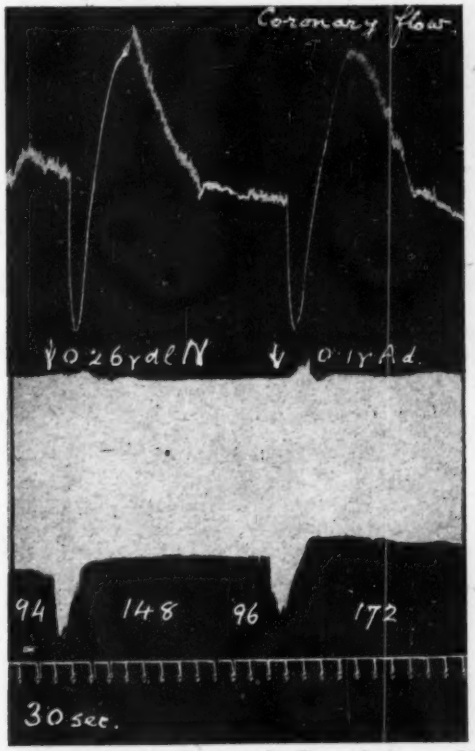

FIG. 6.—Cat heart, Langendorff perfusion. Upper record was coronary flow by Stephenson's method; middle record was amplitude of contractions; heart rate given in figures below. The effects of injecting into the cannula 0.26 μg. *dl-nor*adrenaline and of 0.1 μg. adrenaline were similar on the coronary flow and amplitude. *Nor*adrenaline had a smaller action on the heart rate.

increased it by 76 beats per min. When half these doses were used, the effect on the rate was the same for both, and, as there was less increase in force of contraction, the initial diminution of coronary flow was absent. Marsh, Pelletier, and Ross (1948) also observed that arterenol had the same effect on the rate, contraction, and coronary flow as *dl*-epinephrine in the perfused rabbit and cat heart. In the heart-lung preparation of the dog, observations were very kindly made for us by our colleagues Drs. J. M. Walker and E. M. Lourie in the course of another investigation. Fig. 7 shows the similar effect of 2 μg. *l*-adrenaline

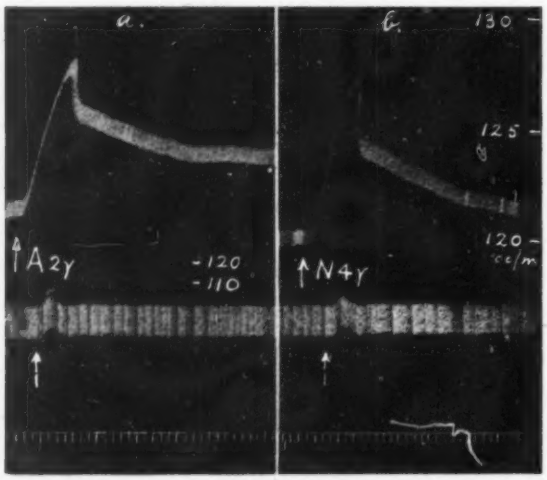

FIG. 7.—Heart-lung preparation of dog. Upper record is the outflow from the coronary sinus collected by a Morawitz cannula and recorded with Stephenson's recorder. Lower record is pressure in brachiocephalic artery. In (*a*) the dilator effect of injecting 2 μg. adrenaline into the s. vena cava is shown, and in (*b*) the similar effect of 4 μg. *dl-nor*adrenaline. Note that the effect of adrenaline was more prolonged. (Experiment of J. M. Walker and E. M. Lourie.)

and of 4 μg. *dl-nor*adrenaline on the outflow from the coronary sinus collected by a Morawitz cannula and recorded by Stephenson's method (1949). The record shows that the effect of adrenaline was more prolonged; this was repeatedly observed. Folkow, Frost, and Uvnäs (1949) have also found that *nor*adrenaline exerts a dilator action on the coronary vessels of the dog.

Renal vessels.—Because renal ischaemia causes hypertension, we examined the effect of *nor*-adrenaline on renal blood flow to see if it caused a greater diminution than adrenaline. The action was studied in cats under chloralose anaesthesia in which the adrenal glands were excluded from the circulation and to which an injection of heparin had been given. A cannula tied into one renal vein conducted the blood through a rubber tube back into the external jugular vein, and the rate of flow through the tube was measured by collecting the blood by a T-piece in the tube during a period of 10 or 15 sec. In one experiment the blood pressure was maintained constant by connecting the femoral artery to a reservoir of blood under an air pressure equal to that in the blood. The results, shown in Table II, indicated that *nor*-adrenaline has not a greater constrictor action on the renal vessels than adrenaline, but that, if anything, the constrictor action of *nor*adrenaline is less. This result is in agreement with that of Gaddum, Peart, and Vogt (1949) on the perfused renal vessels of the rabbit. They found that *l-nor*-adrenaline was equal in constrictor action to *l*-adrenaline when given in 2.5 times as great a dose.

TABLE II

COMPARISON OF CONSTRICTOR ACTION OF ADRENALINE AND *nor*ADRENALINE IN RENAL VESSELS

	Venous outflow c.c./sec.		Fall in flow c.c./sec.
	Before	After	
16 μg. *dl-nor*adrenaline ...	0.47	0.33	0.14
8 μg. *l*-adrenaline ...	0.6	0.38	0.22
4 μg. *l*-adrenaline ...	0.72	0.59	0.13
8 μg. *dl-nor*adrenaline ...	0.92	0.82	0.10
4 μg. *l*-adrenaline ...	1.02	0.88	0.14

In another experiment the renal blood outflow was measured during the continuous intravenous infusion first of adrenaline and then of *nor*-adrenaline; in this experiment a compensating reservoir to maintain a constant blood pressure was not used. The rate of infusion was chosen so that the renal flow could be measured at the same blood pressure with the two substances. The results are given in Table III, which shows that the rates of renal flow for a given blood pressure were the same whichever substance was infused.

Summary of results.—The results on the cardiovascular system show that the one important difference between adrenaline and *nor*adrenaline is in the action on the vessels of skeletal muscle, where the former is dilator but the latter constrictor. On the intestinal vessels of the cat, on the coronary vessels of the cat and the dog, on the vessels of the rabbit ear during perfusion with 2-benzylimidazoline, *nor*adrenaline is dilator when adrenaline is dilator. No evidence was found that

TABLE III

RENAL BLOOD FLOW

Substance	Rate of infusion μg./min.	Duration of infusion min.	Height of blood pressure mm.	Renal flow c.c./min.
l-adrenaline ...	3.4	10	150	0.5
,, ...	3.4		140	0.53
,, ...	3.4		130	0.47
*dl-nor*adrenaline	15.4	11	136	0.47
,,	15.4		128	0.47
,,	15.4		124	0.48
l-adrenaline ...	7.5	6	136	0.45
,, ...	7.5		120	0.47

*nor*adrenaline has a greater constrictor action than adrenaline on the renal vessels.

The effect of denervation

The nictitating membrane.—The normally innervated nictitating membrane of the cat is much less affected by *nor*adrenaline than by adrenaline, but after removal of the superior cervical ganglion and postganglionic degeneration the membrane becomes equally sensitive to both substances. Thus nerve degeneration brings about a small increase in sensitivity to adrenaline, but a large increase to *nor*adrenaline (Bülbring and Burn, 1949).

The pupil dilator muscle.—We have examined the changes produced by degeneration of the sympathetic fibres to the iris. About eight days after extirpation of the ganglion on one side, the cat was anaesthetized with urethane, and the pupils were examined under a bright light during injection of adrenaline and of *nor*adrenaline. The minimum amounts of each substance which caused a dilatation of (*a*) the denervated and (*b*) the normal pupil were determined, and results in three cats are given in Table IV, which shows

TABLE IV

RELATION OF ACTION ON NORMAL AND DENERVATED PUPIL

Cat	Minimal dilating dose	Normal μg.	Denervated μg.	Ratio
1	*l*-adrenaline	2	1	2
	*l-nor*adrenaline	30	1	30
2	*l*-adrenaline	2	0.1	20
	*l-nor*adrenaline	25	0.4	62
3	*l*-adrenaline	7.5	0.5	15
	*l-nor*adrenaline	100	2.0	50

that the dose calculated in terms of *l-nor*adrenaline just sufficient to dilate the normal pupil was found to be 12–15 times greater than for *l*-adrenaline. Denervation increased the sensitivity to *nor*adrenaline much more than to adrenaline, and in one cat the denervated pupil was equally sensitive to both. The behaviour of the pupil was thus similar to that of the nictitating membrane. It was always observed that injections of *nor*adrenaline, too small to cause dilatation of the normal pupil, caused constriction of the pupil.

Greer, Pinkston, Baxter, and Brannon (1938) have also investigated the action of adrenaline and *nor*adrenaline on the normal and on the denervated pupil. They wished to compare the effect of *nor*adrenaline with that of hepatic nerve stimulation, and they drew no conclusions about the modification of the pupil response by denervation. Their photographs, however, agree with our findings.

Action in other organs

The spleen.—The relative activity of adrenaline and *nor*adrenaline in causing contraction of the cat spleen perfused *in situ* has been stated by Gaddum, Peart, and Vogt (1949) to be 0.5–1.0, this being the ratio of the dose of *l-nor*adrenaline to *l*-adrenaline. Our results in spinal cats using a spleen plethysmograph gave a wider range. Thus

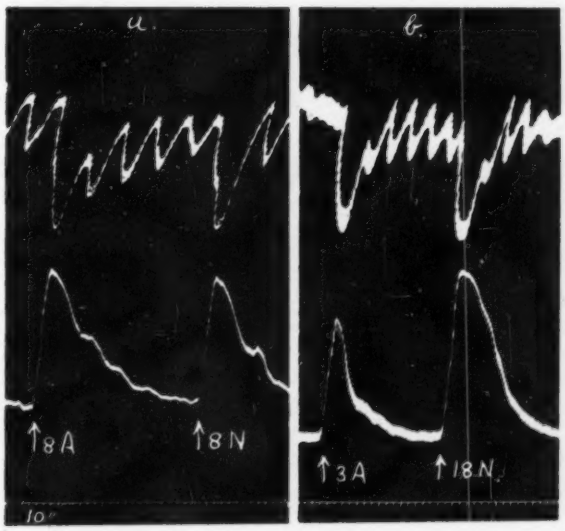

FIG. 8.—Cat, spinal preparation. Upper record spleen volume, lower record blood pressure. The figure illustrates the decline in sensitivity to *nor*adrenaline, and the increase in sensitivity to adrenaline. Early in the experiment 8 μg. adrenaline was equal in action to 8 μg. *dl-nor*adrenaline. Late in the experiment 3 μg. adrenaline was equal to 18 μg. *dl-nor*adrenaline.

in one cat *l-nor*adrenaline was found to be twice as active as *l*-adrenaline, and in another only one-fifth as active. Even in the same cat the relative potency changed in the course of the experiment, *dl-nor*adrenaline decreasing from equivalence to *l*-adrenaline to one-sixth the potency. The initial and final observations in this experiment are shown in Fig. 8. While the sensitivity to *nor*-adrenaline greatly declined, that to adrenaline slightly increased. The various results in different experiments are given in Table V.

TABLE V

CONSTRICTOR EFFECTS IN SPLEEN

Cat	Equivalent doses in μg.		Ratio *l-nor*adr./*l*-adren.
	*dl-nor*adr.	*l*-adren.	
1	20	2	5.0
2	8	3	1.3
3	3	1	1.5
4(*a*)	8	8	0.5
4(*b*)	18	6	1.5
4(*c*)	18	3	3.0
5	80	10	4.0
6	10	1.25	4.0
7	10	1.8	2.7
			Range 0.5–5.0

Bronchioles.—At least two comparisons on the bronchioles have already been made. Tainter, Pedden, and James (1934) found that *dl-nor*-adrenaline had one-seventh of the activity of adrenaline in perfused guinea-pig lungs in relaxing

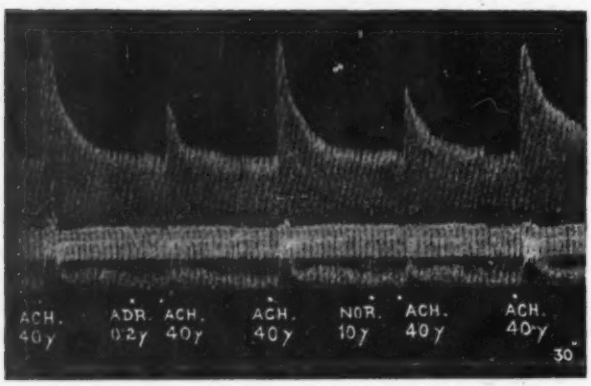

FIG. 9.—Bronchiolar constriction recorded by method of Konzett and Roessler in guinea-pig anaesthetized with urethane. Figure shows constrictor action of 40 μg. acetylcholine, and also the diminished action when 0.2 μg. adrenaline was injected beforehand. The effect of this amount of adrenaline is shown to be slightly greater than the effect of 10 μg. *nor*adrenaline. (Experiment of N. K. Dutta.)

TABLE VI

AMOUNTS WHICH INHIBIT BRONCHOCONSTRICTION EQUALLY

Exp.	Constrictor agent	*l*-adren. μg.	*dl-nor*adren. μg.	Ratio $\frac{l\text{-}nor.}{l\text{-}adr.}$
1	Histamine	10.0	>40	>2
2	,,	0.5	>20	>20
3	,,	0.2	>80	>200
4	,,	5.0	120	12
5	Acetylcholine	0.2	15	37.5

the contraction caused by histamine or pilocarpine. Luduena, Ananenko, Siegmund, and Miller (1949) found a smaller activity—namely, that *l-nor*-adrenaline had one-seventeenth the activity of adrenaline. Our thanks are due to our colleague Dr. N. K. Dutta for making comparisons in anaesthetized guinea-pigs by the method of Konzett and Roessler (1940). The result in one experiment is shown in Fig. 9 and the different results are given in Table VI.

In these experiments there is again evidence of a very variable relationship between adrenaline and *nor*adrenaline. In all experiments *nor*-adrenaline was weaker than adrenaline as a bronchodilator, but it was difficult to quote a single figure as expressing the mean. Some unknown factor appeared to modify the relative activity.

Inhibition of cat intestine.—Many workers have used the inhibitory action of *nor*adrenaline on isolated loops of rabbit intestine as a means of estimation. The inhibition of the movements of the cat intestine *in situ* by *nor*adrenaline was observed by Greer *et al.* (1938). They attached considerable importance to it because they felt that it rendered untenable the view that hepatic sympathin or sympathin E was purely motor. We have obtained similar results to those of Greer and his colleagues, an example being given in Fig. 10, in which 5 μg. *dl-nor*adrenaline produced a slightly shorter inhibition of the duodenal contractions than 1.25 μg. *l*-adrenaline.

Effect in skeletal muscle.—Adrenaline has an action in skeletal muscle which was demonstrated by Bülbring and Burn (1942). If the sciatic nerve of a cat (anaesthetized with chloralose) is stimulated by maximal single shocks at rates up to 15 per min., the tension developed by each twitch of the gastrocnemius can be recorded on the drum. When a dose of neostigmine, too small by itself to modify the tension, is first injected a following injection of adrenaline causes an increase in tension. An example of this action is shown in

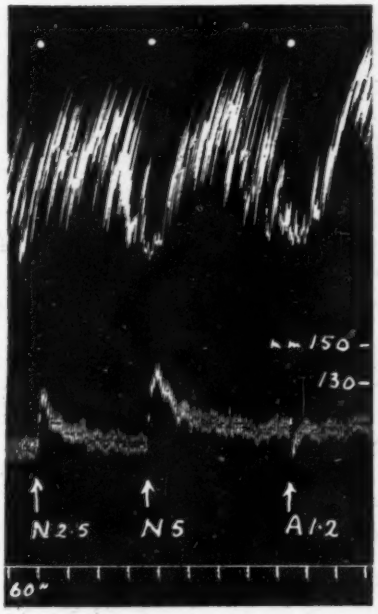

FIG. 10.—Cat, chloralose. Upper record of volume of balloon in the duodenum. Lower record of blood pressure. At N 2.5, 2.5 μg. *dl-nor*adrenaline; at N 5, 5.0 μg. *dl-nor*adrenaline; at A 1.2, 1.25 μg. adrenaline was injected intravenously. Note that N 5 produced slightly shorter inhibition than A 1.2.

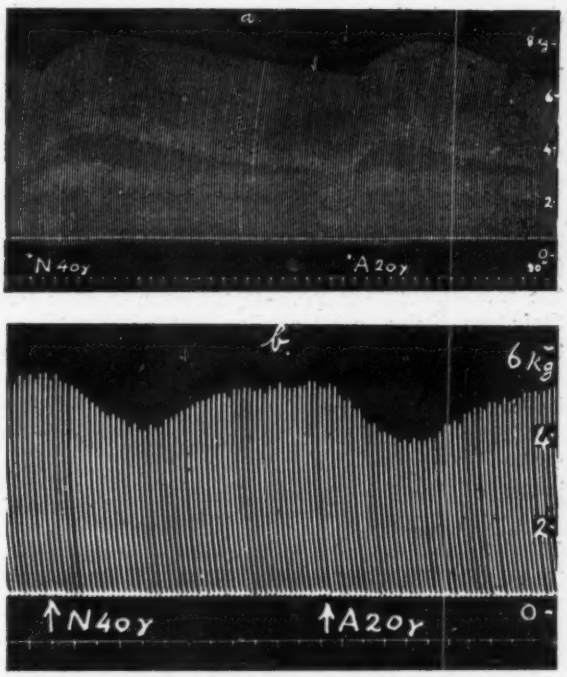

FIG. 11.—Cat, chloralose. Record of tension in gastrocnemius muscle in response to maximal single shocks applied to the sciatic nerve. (*a*) Shows the increase of tension produced by injecting first 40 μg. *dl-nor*adrenaline, and later 20 μg. adrenaline into the iliac artery. These injections were made 30 min. after the injection of 20 μg. neostigmine. (*b*) Shows a record in another cat in which the same injections caused a decrease in tension. These injections were made shortly after the injection of 30 μg. neostigmine. *Nor*adrenaline and adrenaline produce the same effect on the muscle tension.

Fig. 11*a* in which an identical effect exerted by *nor*adrenaline is also seen. When larger amounts of neostigmine are given, the injection of adrenaline may have the opposite effect, causing a decline in the tension. In these circumstances, too, *nor*adrenaline has the same action (Fig. 11*b*).

DISCUSSION

Adrenaline causes vasodilatation in two conditions; first in the cat under ether when the blood pressure is high because the vagi have been cut, and second in the cat to which ergotoxine or some other reversing agent has been given. There has always been discussion whether these two conditions are essentially the same. Our observations appear to indicate that they are not, because the observations show that *nor*adrenaline does not cause vasodilatation in the cat under ether, whereas it causes dilatation in the rabbit ear vessels in the presence of a reversing agent exactly as does adrenaline.

The vascular action of *nor*adrenaline thus differs from that of adrenaline because it does not dilate the vessels of the denervated hindleg of the cat, but rather it constricts them. The difference is probably confined to the muscle vessels. The action of *nor*adrenaline is similar to that of adrenaline (in similar doses) in dilating (*a*) the coronary vessels of the cat and the dog, (*b*) the intestinal vessels of the cat, and (*c*) the vessels of the rabbit ear in the presence of a reversing agent.

Denervation, in some tissues at least, produces a greater change in the reaction to *nor*adrenaline than to adrenaline. Bülbring and Burn (1949) demonstrated that this was true for the nictitating membrane, and used the observation to estimate the relative amounts of adrenaline and *nor*adrenaline present in a mixture. Our results show that the same change occurs in the reaction of the pupil. Only when large amounts of *nor*adrenaline are injected does the normal pupil dilate; but the denervated pupil is about equally sensitive to *nor*adrenaline and to adrenaline.

These observations prompt the suggestion that in some tissues the presence of the sympathetic nerve supply protects the end-organ against the action of *nor*adrenaline in the blood stream, though it

does not similarly protect the end-organ against adrenaline. If *nor*adrenaline is the chemical transmitter of the nervous impulse, such a protection might be useful from a teleological standpoint, as it would enable the nerve to have sole control of the end-organ except for the emergency when adrenaline was liberated.

The great increase in sensitivity to *nor*adrenaline after denervation may explain West's observation (1947) that pieces of intestine or uterus stored in the refrigerator for periods up to five days lose their sensitivity to adrenaline but do not lose it to *nor*adrenaline. Perhaps two processes go on side by side: a general failure of the tissue metabolism measured by the loss of sensitivity to adrenaline; a failure of the mechanism of innervation leading to a denervation and therefore an increase in sensitivity to *nor*adrenaline.

If such a protection is afforded by innervation against the effect of *nor*adrenaline, it evidently plays little part in the blood vessels, the heart, the spleen, and the intestines, though this is a problem for further investigation. Certainly the spleen may become increasingly insensitive to *nor*adrenaline in the course of a few hours in spite of the opposite change taking place in the reaction to adrenaline.

Summary

1. An important difference between the vascular action of *nor*adrenaline and that of adrenaline is that, whereas the latter causes dilatation of the vessels of the denervated hindlimb of the cat, the former causes constriction. This difference is in the muscle vessels.

2. *Nor*adrenaline like adrenaline dilates the coronary vessels of the cat and dog and in small doses also the intestinal vessels. In the vessels of the rabbit ear, the constrictor action of *nor*adrenaline is just as easily converted to a dilator action by 2-benzylimidazoline as is that of adrenaline.

3. Denervation increases the action of *nor*adrenaline on the nictitating membrane and on the pupil much more than it increases that of adrenaline. We suggest that some mechanism exists to protect the nictitating membrane, the pupil, and probably other organs from *nor*adrenaline in the blood; this protection is less effective against adrenaline. When the nerves degenerate the protection disappears.

4. *Nor*adrenaline causes contraction of the spleen *in situ*. During the course of an experiment the spleen becomes less sensitive to *nor*adrenaline though it increases slightly in sensitiveness to adrenaline.

5. *Nor*adrenaline has a smaller constrictor action on renal blood flow than adrenaline.

6. *Nor*adrenaline inhibits intestinal movements recorded by a balloon in the duodenum. Its inhibitory action appears to be about half that of adrenaline, though the relation probably varies.

7. *Nor*adrenaline has the same effect as adrenaline on skeletal muscle previously treated with neostigmine, no matter whether the effect of adrenaline is to augment or to diminish the tension developed when shocks are applied to the sciatic nerve.

8. *Nor*adrenaline has much less effect than adrenaline in dilating the bronchioles, but the quantitative relation varies considerably.

We wish to thank Dr. N. K. Dutta for carrying out the experiments on the bronchioles and Mr. St. John Ives for doing many of the experiments on the rabbit ear. The work was done during the tenure by one of us (D.E.H.) of a Medical Research Fellowship awarded by the National Research Council of Canada.

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THE PHARMACOLOGICAL ACTIONS OF POLYMETHYLENE BISTRIMETHYLAMMONIUM SALTS

BY

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Since the classical work of Crum Brown and Fraser, the curariform action of many onium salts has been recognized, and other activities exerted by these compounds have been described, such as those commonly called nicotine-like or muscarine-like. In the present paper an account is given of the actions of members of a polymethylene bistrimethylammonium series, whose general formula is $(CH_3)_3\ N^+(CH_2)_n N^+(CH_3)_3.2I$. The compounds will be referred to by the value of n; thus C10 is the decane derivative, where $n=10$. Our attention was drawn to the series during a test of the power of the octamethylene compound to liberate histamine. After the injection of a small dose into a cat, there was no depressor response, such as histamine-liberators cause (MacIntosh and Paton, 1949); on the contrary, the blood pressure rose. This rise was evidently asphyxial, since the respiration had simultaneously ceased; but there were no gasps or convulsive movements such as usually accompany asphyxia, and the sequence of events suggested some form of neuromuscular block.

This series has also been independently studied by Barlow and Ing, with whom a simultaneous preliminary report was arranged (Barlow and Ing, 1948a; Paton and Zaimis, 1948a), and who have now reported their results more fully (Barlow and Ing, 1948b).

Following a further note (Paton and Zaimis, 1948b), preliminary clinical trials of the decane derivative as a substitute for *d*-tubocurarine chloride in anaesthesia and convulsion therapy were instituted. These have proved successful, and the name "decamethonium iodide" has been approved by the British Pharmacopoeia Commission as the official name for the compound.

Our investigations of the pharmacological actions of these compounds have been chiefly directed to studying their activity in blocking neuromuscular transmission. Particular attention has been paid also to describing how the pharmacological activity varies with the length of the polymethylene chain. Later papers will report more fully experiments on other pharmacological actions.

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METHODS

Effects on neuromuscular transmission

Cats anaesthetized with chloralose (80 mg./kg.), after induction with ether, were used in most of the experiments. For recording the tension of muscle twitch, tibialis anterior was prepared; the preparation was mounted on the Brown-Schuster myograph stand, and an isometric steel spring myograph was used for recording on the smoked drum. The muscle was excited either by slightly supra-maximal shocks to the sciatic nerve through shielded silver electrodes, or directly by induction coil break shocks. The nerve was tied centrally, above the point of stimulation and above the point of entry of its blood supply. Injections were made either intravenously through a cannula tied into the femoral or jugular vein, or by the method of close arterial injection into the anterior tibial artery (Brown, 1938a). A few animals were anaesthetized with pentothal infused at a rate of 0.3–0.5 mg./min., or with ether from an "Oxford vaporizer" adapted for animal use, using 6–7 per cent ether.

For experiments on unanaesthetized animals, injections of volumes of 0.01 c.c./g. were made into the tail vein of mice (white, male, weight 18–20 g.) and rats (male, weight 100–150 g.). (The effective dose was that which prevented the animal righting itself when placed on its back.) Rabbits (male, weight 1.5–2 kg.) were used for the continuous infusion head-drop method (Dutta and MacIntosh, 1949), or received rapid intravenous injections in the marginal ear vein. Cats, monkeys (*Macaca mulatta*), and a baboon (*Papio anubis*) received injections in the saphenous vein. Frogs were tested as described by King (1935).

Other methods

In cats anaesthetized with chloralose, the contraction of the nictitating membrane was recorded on the smoked drum. The membrane was excited to contraction by maximal stimuli applied to the peripheral

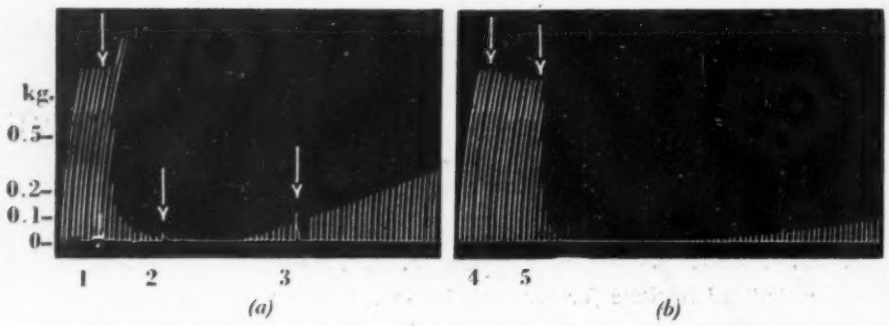

FIG. 1 (*a*).—Cat, chloralose, 3.7 kg. Record of contractions of tibialis excited by supramaximal shocks to the sciatic nerve every 10 sec. At 1, 0.12 mg. C10 intravenous injection. At 2 and 3, tetanic stimulus to motor nerve, 50/sec. (*b*) Same experiment, 36 min. later. *d*-Tubocurarine chloride 0.3 mg. intravenously 5 min. previously. At 4, 0.12 mg. C10 i.v. At 5, 0.24 mg. C10 i.v.

stump of the cervical sympathetic, cut and separated from the vagus in the neck ; a rate of stimulation of 10 per sec. produced a well-sustained contraction. In other experiments, the isolated rabbit intestine preparation described by Feldberg and Lin (1949) was employed.

Muscarine-like action was tested on rabbit or guinea-pig small intestine, suspended in Ringer solution containing magnesium chloride (0.004 g./100 c.c.) : atropine sulphate (10^{-7}) was used as an antagonist. The frog's rectus abdominis suspended in frog Ringer solution was employed in customary fashion to test for nicotine-like stimulation of skeletal muscle. The spinal cat was prepared as described by Barger and Dale (1910) for the detection of pressor activity. Anticholinesterase activity was determined using a Warburg manometer, with rabbit's laked washed red cells and acetyl-β-methylcholine (0.027 *M*) or rabbit plasma and benzoyl choline (0.0055 *M*) as sources of and substrates for " true " or " pseudo " cholinesterase respectively. The substrate and inhibitor (if any) were placed in the sidearm of the Warburg bottle, so that shaking brought them into simultaneous contact with the enzyme ; readings of the manometer were then made every 10 minutes for two hours. The surface tension of aqueous solutions of the compounds against air was measured with a De Nöuy tensiometer. Values for the surface tension of glass-distilled water of 71.5–72.1 dynes/cm. at 20–23° C. were obtained.

Actions on the respiration were recorded at first by discharging the expirations of the animal (by means of light rubber valves) into a large glass vessel from which a fine adjustable leak was provided, and measuring the pressure within it by a sensitive tambour ; later measurements were made with the respiration recorder described by Paton (1949a). Blood pressure was measured in the usual way, a cannula coated with silicone and filled with saline containing heparin being inserted into the carotid artery. We are much indebted to Dr. J. A. B. Gray for recording action potentials from the peroneal nerve and tibialis muscle for us in certain experiments.

RESULTS

Neuromuscular block

Action of C10 on the neuromuscular junction

The most notable activity of this series is that possessed by the higher members in causing neuromuscular block. Fig. 1*a* shows the effect of the intravenous injection of a small dose of C10 (the most active compound in this respect) on the contractions of cat's tibialis muscle excited through its motor nerve. At first the tension is increased, and between the contractions fasciculations of the muscle can be seen. (The other muscles of the animal also exhibit these incoordinated contractions.) Then the twitch tension begins to diminish until, with this dose, the muscular contraction is almost completely abolished. When the muscle is completely paralysed to stimulation through its nerve, it is still capable of responding to direct stimulation (Fig. 2).

During the progress of such a paralysis the action potential of the motor nerve to tibialis remains

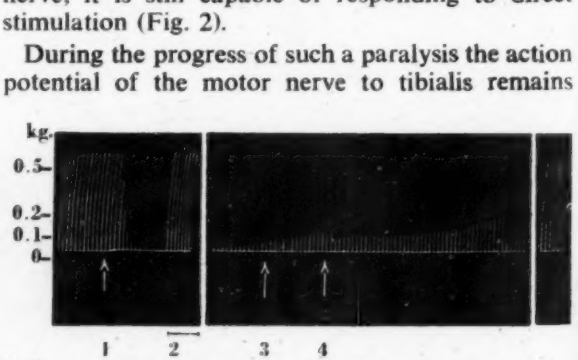

FIG. 2.—Cat, chloralose, 2.6 kg. Tibialis; nerve shock every 10 sec. At arrow 1, injection of 0.1 mg. C10 i.v. During 2, direct stimulation of muscle. At 3, 1 mg. atropine sulphate i.v. At 4, 0.5 mg. neostigmine methylsulphate i.v.

completely unimpaired while the muscle action potential and twitch tension dwindle and disappear (Fig. 3A). The site of paralysis, therefore, must be placed in the end-plate region or in the terminal nerve endings.

One possible mechanism for such a paralysis might be abolition of the release of acetylcholine by the nerve endings, as has been described for procaine (Harvey, 1939) and suggested for atropine (Brown, 1937). We have found, however, that the effects of acetylcholine given by close arterial

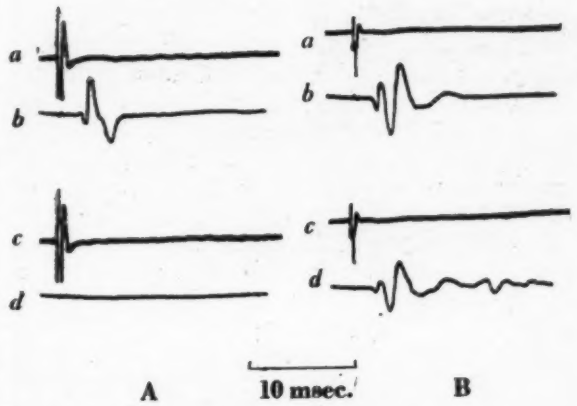

FIG. 3 (A).—Cat, chloralose. Supramaximal shock to sciatic nerve every 10 sec. (*a*, *c*) Record of action potential of peroneal nerve (preceded by stimulus artifact), (*a*) before C10 ; (*c*) after 100 μg. C10/kg.: and (*b*, *d*) record of action potential of tibialis muscle, (*b*) before C10; (*d*) after C10. Time = 10 msec. (B) Cat, chloralose, 2.6 kg. Record as in (A) showing repetitive muscle action potential after 50 μg. C10.

injection are antagonized as much as (or more than) the effect of a nerve volley by a dose of C10, just as they are by curare (Fig. 4). A similar suppression of the response to acetylcholine is shown in Fig. 9.

The blocking action of C10 therefore cannot be explained by an interference with release of acetylcholine. Further, since these compounds are onium salts and do not have any local anaesthetic potency, there is no reason to expect a procaine-like action. Since the block is completely reversible, there is no reason to suspect any action such as that due to botulinus toxin. Finally, the possibility that acetylcholine liberation might be depressed was tested directly in one experiment, in which the tibialis anterior muscle of a cat was dissected and perfused in isolation with eserinized cat's plasma. The plasma was then tested on the cat's blood pressure. During the control period, there was no detectable depressor activity in the effluent ; stimulation of the motor nerve at a rate of 50 per sec. for 2 min. caused the release of depressor material in a concentration equivalent to 5 mμg. acetylcholine per c.c. in the effluent, and the activity of this depressor material was abolished by the injection of 0.5 mg. atropine into the assay cat ; this release was not prevented by adding C10 to the perfusion fluid to a concentration of 10^{-5}.

The fasciculations and the potentiation of the twitch preceding neuromuscular block led us to test these compounds for anticholinesterase activity, since known anticholinesterases produce similar actions ; and experiments in this connexion are described below which revealed that C10 and its neighbours possess some activity of this kind. (Later work (Zaimis, 1949) indicates, however, that if this anticholinesterase action plays a part in causing these effects, such a part is small.) The potentiation of the twitch may be considerable, and is best seen with smaller doses of C10, such that the subsequent neuromuscular block is too small to obscure the potentiating process ; Fig. 5 exemplifies such an experiment. With still smaller doses, a transient enhancement of the twitch tension may be the only evidence that C10 has been injected. It was necessary, therefore, to test whether the neuromuscular block might even be a direct consequence of this anticholinesterase activity or not.

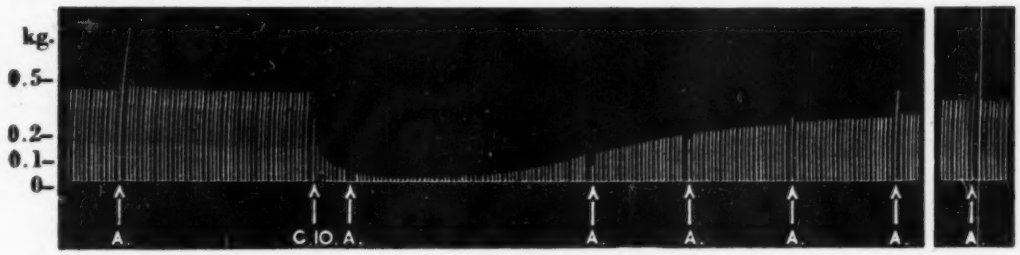

FIG. 4.—Cat, chloralose, 2.8 kg. Tibialis: nerve shock every 10 sec. At A, injections of 5 μg. acetylcholine intra-arterially. At C10, 6 μg. C10 injected intra-arterially.

It is well known (Briscoe, 1936 ; Rosenblueth, Lindsley, and Morison, 1936) that eserine and other anticholinesterases can cause neuromuscular block, which has been ascribed to the presence of an excess of acetylcholine in the region of the neuromuscular junction. Under such conditions, however, the interposition of a tetanus, or the close arterial injection of acetylcholine

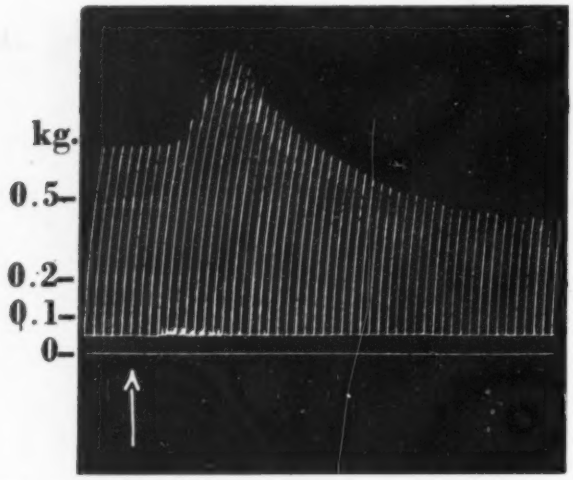

FIG. 5.—Cat, chloralose, 2.6 kg. Tibialis; nerve shocks every 10 sec. At arrow, 26 μg. C10 injected intravenously. The same dose was given 5 min. previously without any effect.

increases the block for the succeeding twitches (Bacq and Brown, 1937). We have used this phenomenon as a test of the nature of the block caused by C10. Figs. 1*a* and 4 show that there is no such depressant action by a tetanus or by injection of acetylcholine respectively on the twitch of a muscle partially paralysed with C10; and it has been our constant experience that it would be hard to judge from the subsequent twitches that a tetanus had been applied or an injection made. (An apparent slight deepening of the block by acetylcholine in Fig. 9 was due to traces of C10 from the previous injection.) We have, moreover, never observed any relation between the rate of stimulation and the development of the block. Indeed, with a large dose of C10 almost complete paralysis may occur after a single twitch. It is unlikely, therefore, that block due to C10 is the result of the accumulation of acetylcholine at the end-plate. We do not wish, however, to underestimate the resemblance of some of our tracings to those resulting from injections of potent antiesterases (cf. Brown, Burns, and Feldberg, 1948), but it is possible that some of the latter produce a block otherwise than by their antiesterase action.

Our experiments in this connexion also showed that a muscle could still maintain a tetanic contraction at a height comparable with the twitch tension when partially paralysed with C10. It is well known that the curarized muscle cannot do this; our experiments on this important difference will be reported separately.

Duration of action

In the anaesthetized cat, the complete or nearly complete paralysis of tibialis that follows an intravenous dose of 30 μg./kg. of C10 usually begins to recover in 5–10 min., and recovery is complete in about 15 min. Sometimes a twitch tension greater than the initial may be observed for a few minutes of the recovery (recapitulating the initial potentiation) before it returns to the original level. The presence of the drug, however, is still detectable for some time after the twitch tension has returned to normal, since the same dose given again less than 30 min. after the first injection produces a greater effect. But with suitable spacing of doses, reproducible cycles of paralysis and complete recovery can be obtained for many hours, the only important change commonly observed being a diminution of the initial potentiation with the lapse of time. Our experience also suggests that C10 has a rather steep dose-response curve; thus, a dose of 20 μg./kg. was sometimes without visible effect on twitch tension in an animal in which 30 μg./kg. produced temporarily a complete block. Corresponding to this, recovery from a C10 block, once it starts, is often rather rapid. *d*-Tubocurarine chloride differs significantly both in having a longer duration of action for a given peak action and in the slower waning of its effects.

Similar time relations are observed in unanaesthetized cats and in rabbits; both animals, after a dose of C10 sufficient to paralyse them fully, recover in about 10 min., whereas *d*-tubocurarine chloride has a somewhat more prolonged action. In the monkey, however, the reverse is the case, and with equally effective doses C10 has a duration of action about two to three times longer than that of *d*-tubocurarine chloride.

We have given C10 by other routes in a few experiments. Administered by stomach tube, C10 is ineffective in cat and rabbit in doses less than fifty times the effective intravenous dose, but a dose of a hundred times may be lethal in an animal starved for 24 hours. The paralysis takes an hour or more to appear. By the subcutaneous route in the rabbit, about three times the intravenous dose is required for equal maximum effects, and the paralysis does not appear for about 10 min. nor disappear entirely for about 2 hours. With intramuscular injections, slightly smaller doses are required, and the onset of paralysis is quicker. We have not, however, studied the relative doses required for equal peak effects by the various routes in any detail, and the values quoted are only approximate.

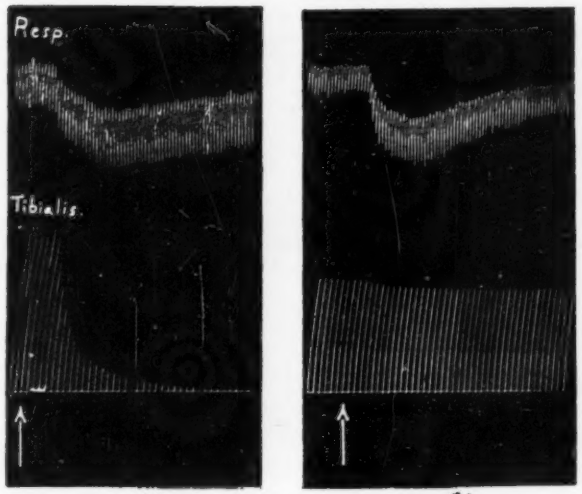

FIG. 6.—Cat, chloralose. Record of respiration and of tibialis; nerve shocks every 10 sec. (*a*) At arrow, 0.1 mg. C10 intravenously. (*b*) At arrow, 0.2 mg. *d*-tubocurarine chloride intravenously.

Action on the respiration

Fig. 6*a* is the record of an experiment in which the respiratory volume was recorded simultaneously with the response of the tibialis muscle to single nerve shocks. Although the muscle response was almost completely abolished, the respiratory volume was but slightly affected. This has been a constant and striking experience. Sometimes, indeed, an increase in the respiratory minute volume has been observed ; a counterpart, perhaps, of the phase of potentiation of the muscle twitch which has been already mentioned. With larger doses of C10, respiratory depression can of course be induced ; but it is again remarkable how much sooner recovery of adequate respiration takes place than recovery of the normal muscle twitch.

In Fig. 6*b* is also shown the record of a similar experiment with *d*-tubocurarine chloride. In contrast to C10, distinct respiratory depression was produced, with an almost negligible effect on the tibialis twitch. Our experience has been consistently of this kind, that with *d*-tubocurarine chloride, respiratory depression accompanies or even precedes paralysis of the tibialis twitch.

The contrast between the two drugs in this respect appeared so striking that it will be reported more fully elsewhere.

Effect of varying the anaesthetic

Although most of our experiments were made with cats anaesthetized with chloralose, we have also used ether alone and pentothal alone. In the one experiment with pentothal anaesthesia, C10 appeared to be somewhat more effective than with chloralose, 20 μg./kg. being adequate for complete abolition of the tibialis twitch, although respiration was only slightly depressed with the above dose. No preliminary potentiation or fasciculations were seen.

With ether (6–7 per cent), on the other hand, C10 was less effective than with chloralose, and the fully paralysing dose of C10 varied from 40 to 70 μg./kg. in four animals. Potentiation of the twitch and fasciculations were never seen, even with only feebly paralysing doses. Sparing of respiration was much less prominent than in the animal anaesthetized with chloralose. Tetani were sustained very poorly, as with *d*-tubocurarine

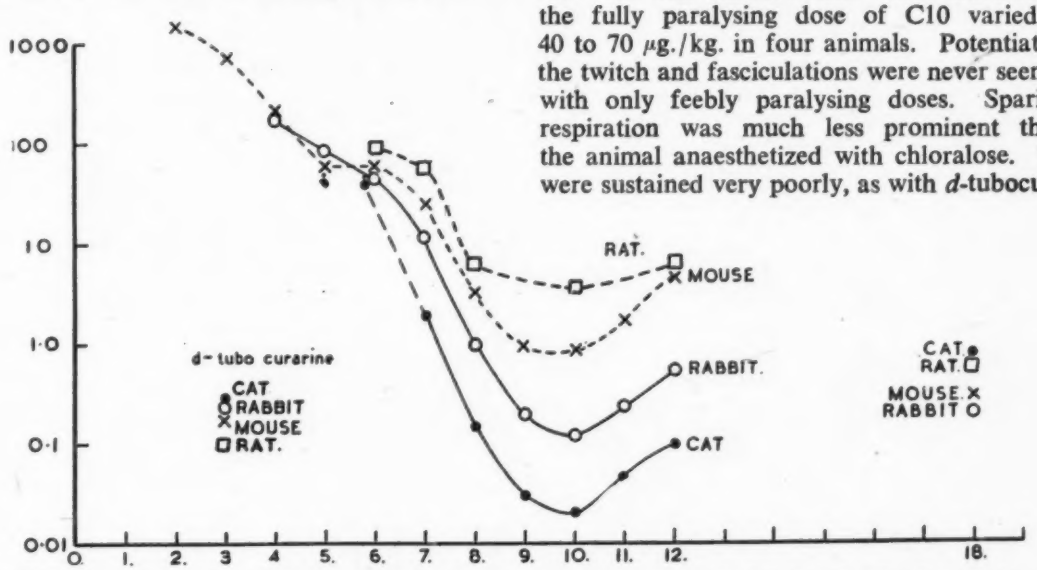

FIG. 7.—Variation of potency of bistrimethylammonium compounds, with varying length of polymethylene chain, and of *d*-tubocurarine chloride, among different species. Abscissa : number of carbon atoms in chain. Ordinate: dose in mg./kg. (Extrapolation of the curves beyond C12 to C18 has not been attempted.)

TABLE I

ACTIVITY OF POLYMETHYLENE BISTRIMETHYLAMMONIUM SALTS IN VARIOUS SPECIES

Compound	Cat	Rabbit		Mouse			Rat		
	(Dose producing 95% paralysis of tibialis) mg./kg.	HDD ± standard error mg./kg.	No. of tests	LD 50 mg./kg.	% Limits of error	ED 50 mg./kg.	% Limits of error	LD 50 mg./kg.	% Limits of error	ED 50 mg./kg.	% Limits of error
C2	—	> 104		1,490	(79–126)	—	—	—	—	—	—
C3	—	> 40		738	(80–125)	559	(75–133)	—	—	—	—
C4	—	161 approx.		246	(23.4–427)	—	—	—	—	—	—
C5	> 40	81.1 ± 11.9	(3)	51.2	(36.272)	—	—	—	—	—	—
C6	> 40	42.4 ± 8.1	(6)	54.8	(90–111)	—	—	96.3	(88.6–113)	—	—
C7	1.9	13.5 ± 2.54	(6)	23.8	(88–114)	—	—	61.3	(81–124)	—	—
C8	0.16	0.919 ± 0.181	(4)	3.37	(88–114)	2.43	(93–108)	6.50	(90–110)	6.14	(90.5–111)
C9	0.036	0.219 ± 0.0187	(14)	0.808	(82–122)	0.709	(72–140)	—	—	—	—
C10	0.030	0.15 ± 0.0058	(65)	0.838	(90–111)	0.691	(87–114)	3.02	(87–115)	2.52	(88–114)
C11	0.060	0.233 ± 0.0121	(4)	1.73	(87–115)	1.33	(89–112)	—	—	—	—
C12	0.10	0.526 ± 0.0300	(6)	4.39	(75–133)	—	—	6.81	(76–131)	5.27	(81–123)
C18	1.5	0.197 ± 0.0260	(4)	0.306	(89–112)	0.306	(89–112)	0.623	(75–133)	—	—
d-Tubocurarine chloride	0.3	0.22 ± 0.0076	(76)	0.167	(92–109)	0.120	(94–106)	0.0964	(86–116)	0.0766	(91–110)
Tetramethylammonium iodide	5	—		—	—	—	—	3.56	(42–237)	—	—

chloride. Indeed, the effects of ether could be said to resemble rather closely those of a previous dose of *d*-tubocurarine chloride, which are described below.

Variation of potency with species

Attempts to estimate the potency of these compounds led at once to the discovery of a very great variation with different species. For the bulk of the experiments different methods of testing were used with different species. Fig. 7 summarizes the results, which are shown in more detail in Table I, together with corresponding figures for *d*-tubocurarine chloride. Further experiments on a few of the compounds showed, however, that variation in the method of testing accounted for only a small part of the species difference. We took as a standard that dose (RD50) which, after injection rapidly by the intravenous route into unanaesthetized animals, causes loss of the ability to right themselves in half the animals. In cats this was very close to the dose required to reduce the twitch tension of tibialis by 95 per cent in the animal anaesthetized with chloralose; in rabbits, it was about 20 per cent less than the head-drop dose (HDD), and it was about 20 per cent less than the LD50 in mice and rats. The figures for monkey were direct estimates of RD50; those for man are based on some preliminary trials (Organe, Paton, and Zaimis, 1949) and represent the dose that made the subjects too weak to sit up or stand or lift any of their limbs. If the activity of C10 in the various species is corrected to this standard, comparable estimates of its potency in these species are obtained, and are shown in Table II. The results of tests on frogs are also included, but it must be remembered that the route of injection used (ventral lymph sac) was quite different from that in the other species.

The actions of these drugs on various species also differed in the manner of the paralysis, and several interesting points emerged. In the monkey, the earliest sign of weakness was an inability to keep the arm above the head; after this appeared progressive weakness of movement and dropping of the head, and only with deep paralysis was the ability to sit up lost. In cats, the paralysis progressed more uniformly, and neck, trunk, and limbs seemed to weaken together; but an unusual and constant feature was the complete relaxation of the nictitating membrane for so long as the paralysis lasted: this relaxation is, indeed, the first sign of paralysis observed after an intravenous injection. Finally, in rabbits, weakness of the hindlegs appeared first, while further paralysis of the limbs and head-drop

TABLE II

RELATIVE POTENCY OF C10 IN VARIOUS SPECIES, AS DOSE REQUIRED TO PARALYSE RIGHTING REACTION

Species:	Cat	Man	Baboon	Rabbit	Monkey	Mouse	Rat	Frog
Dose mg./kg. C10 ...	0.03	0.05	0.14	0.14	0.25	0.691	2.5	10.9
d-Tubocurarine chloride	0.3	0.25	—	0.20	0.09	0.120	0.0766	5.49

followed. The opportunity was taken of administering C10 to one baboon (*Papio anubis*), weight 14.5 kg., which was suffering from a traumatic paraplegia and was to be killed. After the slow injection of C10 at a rate of 0.5 mg./min., head-drop occurred after 2 mg. had been injected, followed by almost complete skeletal paralysis; respiration was still adequate although depressed. Injection of 50 mg. of C5 9 min. later caused partial recovery of arm strength and deepening of respiration.

Variation of potency with length of polymethylene chain

Despite the species variation, C10 was the most potent member of the series by any test for neuromuscular block. Its immediate neighbours in general closely resembled it qualitatively, although not in potency. The steepness of the curve relating potency to chain-length deserves comment; for instance, shortening the polymethylene chain from eight to seven carbon atoms reduced potency more than tenfold. Members of the series remote from C10, however, gave the impression that new activities were appearing. The abrupt change of slope in Fig. 7 in the region of C6 and the attenuation of the species difference with C18 are of great interest. Since head-drop and lethality are not specific tests for neuromuscular block, it is possible that the other pharmacological actions of the series described below become prominent in bringing about an end-point with the members of the series which are relatively inactive in causing neuromuscular block.

A point of interest lies in the activity of tetramethylammonium iodide relative to these compounds. A dose of 5 mg./kg. injected intravenously into a cat produced an effect on tibialis twitch comparable with that of 30–40 μg./kg. of C10. This potency, although small, is greater than that of C4, C5, and C6, which fail to depress neuromuscular transmission in doses of 40 mg./kg. Our results with mice, rats, and rabbits indicate that C2 and C3 are equally or even more inactive in this respect.

Interaction of C10 with d-tubocurarine chloride

Our first attempts to compare the potencies of these two drugs on the cat's tibialis yielded puzzling results, until it was realized that C10 was less effective than usual when *d*-tubocurarine chloride had been given previously. Fig. 1*a* and 1*b* illustrates this point on cat's tibialis. It can also be shown on the rabbit head-drop, and a typical experiment is cited in Table III. In this experiment the preliminary dose of curare was such as caused distinct (although transient) weakness of

TABLE III

EFFECT OF PREVIOUS ADMINISTRATION OF *d*-TUBOCURARINE CHLORIDE (*d*-TC) ON SENSITIVITY TO C10

Cross-over test on four rabbits; 0.15 mg. *d*-tubocurarine chloride per kg. i.v. 15 min. before test

Rabbit HDD (mg./kg.)	
C10 alone	C10 after *d*-TC
0.088, 0.222 } day 1	0.224, 0.365 } day 2
0.104, 0.109 } day 2	0.171, 0.176 } day 1

$$\frac{\text{Dose of C10 after } d\text{-TC}}{\text{Dose of C10 alone}} = 1.81 \text{ (limits of error for P = 0.05: 1.10–2.99)}$$

TABLE IV

EFFECT OF PREVIOUS ADMINISTRATION OF C10 ON SENSITIVITY TO *d*-TUBOCURARINE CHLORIDE

Cross-over test on four rabbits; 0.075 mg. C10 per kg. i.v. 15 min. before test

Rabbit HDD (mg./kg.)	
d-TC alone	*d*-TC after C10
0.264, 0.232 } day 2	0.207, 0.184 } day 1
0.179, 0.231 } day 1	0.131, 0.163 } day 2

$$\frac{\text{Dose of } d\text{-TC after C10}}{\text{Dose of } d\text{-TC alone}} = 0.753 \text{ (limits of error for P = 0.05: 0.723–0.784)}$$

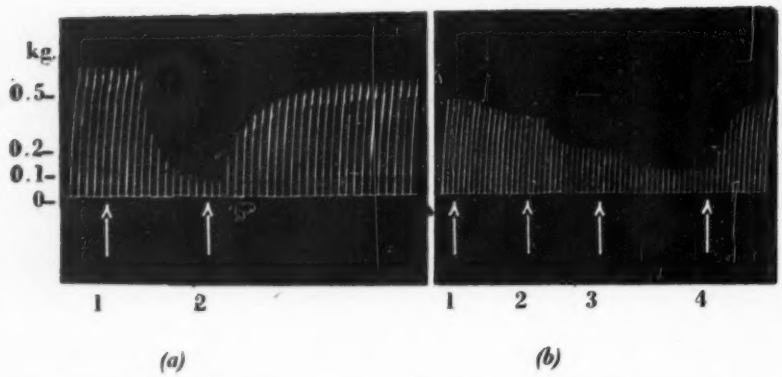

FIG. 8.—(*a*) Cat, chloralose, 2.2 kg. Tibialis: nerve shocks every 10 sec. At (1), 0.1 mg. C10 i.v. At (2), 10 mg. C5 i.v. (*b*) Cat, chloralose, 2.8 kg. Tibialis: nerve shocks every 10 sec. At (1), (2), (3), 1 mg., 0.5 mg., 0.5 mg., respectively, *d*-tubocurarine chloride i.v. At (4), 0.5 mg. neostigmine i.v.

the animals; nevertheless, a larger dose of C10 was required subsequently to produce head-drop. The previous administration of C10 does not (despite its feeble anticholinesterase action) lessen the effect of *d*-tubocurarine chloride, but rather augments it to a slight degree according to the interval between the injections (Table IV).

This antagonistic effect of *d*-tubocurarine chloride is detectable in the rabbit for an hour and in the cat may persist for a similar period. The duration of the antagonism and its effectiveness become greater as the dose is increased. In addition, the fasciculations and potentiation normally produced by C10 are uniformly abolished.

These actions are not specific to *d*-tubocurarine chloride; we have observed them also with its methyl ether, *d*-bebeerine methiodide, N-methyldiaboline iodide, and with tri(diethylaminoethoxy)-benzene triethiodide (" Flaxedil ").

TABLE V

EFFECT OF PREVIOUS ADMINISTRATION OF NEOSTIGMINE METHYL SULPHATE ON SENSITIVITY TO C10

Cross-over test on four rabbits; 0.05 mg. neostigmine methyl sulphate per kg. i.v. 1 min. before test

Rabbit HDD (mg./kg.)	
C10 alone	C10 after neostigmine
0.117, 0.156 } day 1 0.203, 0.121 } day 2	0.112, 0.154 } day 2 0.232, 0.112 } day 1

$$\frac{\text{Dose of C10 after neostigmine}}{\text{Dose of C10 alone}} = 1.00 \text{ (limits of error for P = 0.05: 0.795–1.258)}$$

Antagonists

Eserine and neostigmine are without effect on the neuromuscular paralysis due to C10 and its neighbours. Fig. 2 exemplifies the failure of neostigmine to reverse such block in the tibialis muscle; Fig. 8*b* is an illustration of an effective antagonism by a similar dose to *d*-tubocurarine chloride. (The slight deepening of block due to C10 by prostigmine in this experiment (Fig. 2) was seen at other times, but not constantly.) Eserine was equally ineffective. Similarly with the rabbit head-drop test, a dose of 0.05 mg. neostigmine methyl sulphate per kg., previously given, which increased the HDD of *d*-tubocurarine chloride from 0.316 mg./kg. to 0.615 mg./kg. in four rabbits, did not alter the HDD dose of C10 significantly from 0.149 mg./kg. (Table V).

Observations on the frog's rectus, in which C10 produces a contracture, showed that lower members of the series, inactive both in causing neuromuscular block and in producing a contracture, antagonized this action of C10: C5 and C6 were

TABLE VI

EFFECT OF PREVIOUS ADMINISTRATION OF C6 ON SENSITIVITY TO C10 AND TO *d*-TUBOCURARINE CHLORIDE

Cross-over tests on four rabbits; 10 mg. C6 per kg. i.v. 5 min. before test

Rabbit HDD (mg./kg.)	
C10 alone	C10 after C6
0.100, 0.116 } day 1 0.153, 0.147 } day 2	0.180, 0.273 } day 2 0.240, 0.195 } day 1

$$\frac{\text{Dose of C10 after C6}}{\text{Dose of C10 alone}} = 1.69 \text{ (limits of error for P = 0.05: 1.23–2.41)}$$

d-TC alone	*d*-TC after C6
0.177, 0.336 } day 2 0.266, 0.280 } day 1	0.163, 0.392 } day 1 0.181, 0.227 } day 2

$$\frac{\text{Dose of } d\text{-TC after C6}}{\text{Dose of } d\text{-TC alone}} = 0.878 \text{ (limits of error for P = 0.05: 0.638–1.21)}$$

particularly effective in this respect. This suggested that such antagonism might also exist at the mammalian neuromuscular junction. Fig. 8, a record of an experiment to test the point on the cat's tibialis with C5, shows that this expectation was fulfilled. Table VI summarizes the results of a similar experiment with C6 using the rabbit head-drop method. Table VI also shows that C6 not only has no antagonistic action to *d*-tubocurarine chloride but may even potentiate its action somewhat.

The antagonistic action of C5 (and C6) is complicated to some extent by their ganglionic action (described below), and with large doses there is no doubt that a fall of blood pressure occurs which is due to paralysis of sympathetic vascular tone. This does not, however, affect the recovery of neuromuscular transmission when C5 is administered during a paralysis due to C10. A useful antidotal action can be observed in rabbit and monkey where the dose of C5 is only ten times that of the paralysing dose of C10, and under these conditions vascular effects are trivial. With this ratio of doses, shallow paralyses are cut short, recovery from deeper paralyses is accelerated, and respiratory depression due to larger doses still is greatly lessened. On the cat tibialis preparation, however, a larger ratio of C5 to C10 is usually required, and 3 mg./kg. C5 may be required for a prompt antagonism. The antagonistic action of C5 is easily reversed by increasing the dose of C10, and the renewed onset of neuromuscular block can be again antagonized by further doses of C5; there is a limit to this process, however, and with very large doses of C5 little more recovery from neuromuscular block can be obtained. Similarly, after large doses of C10 it is difficult to demonstrate any antagonism (just as neostigmine is not very effective after large doses of curare).

Potassium has been shown to antagonize the neuromuscular block due to curare (Wilson and Wright, 1936). We have therefore tested it against a similar block due to C10 (Fig. 9*b*). It will be seen that there is no important action, although the dose is enough (when given to the unparalysed muscle) to produce a typical potentiation of the twitch (Fig. 9*a*). Adrenaline appears to be equally ineffective.

Paralysis of autonomic ganglia

A few seconds after the injection of 10 mg. of C6 into a rabbit, we observed that its ears flushed vigorously and became warm, and we have already mentioned the ability of C5 and C6 to cause a fall of blood pressure. The analysis of these effects revealed that these compounds paralyse autonomic ganglia. The evidence for this will be presented in another paper. For the present we wish only to describe the experiments made to compare quantitatively the potencies of

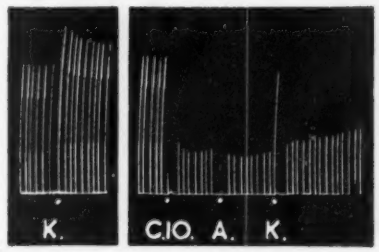

FIG. 9.—Cat, chloralose, 2.8 kg. Tibialis: nerve shocks every 10 sec. Intra-arterial injections: (*a*) At K, 3 mg. KCl. (*b*) At C10, 4 μg. C10; at A, 5 μg. acetylcholine; at K, 3 mg. KCl.

C6 and its neighbours in this respect. The cat's nictitating membrane, excited to a sustained contraction by stimulation of the preganglionic cervical sympathetic trunk at a frequency of 10 shocks per second, provided a useful test for activity of these compounds on transmission in the superior cervical ganglion. Fig. 10 shows a typical tracing in which C5 and C6 and tetraethyl-

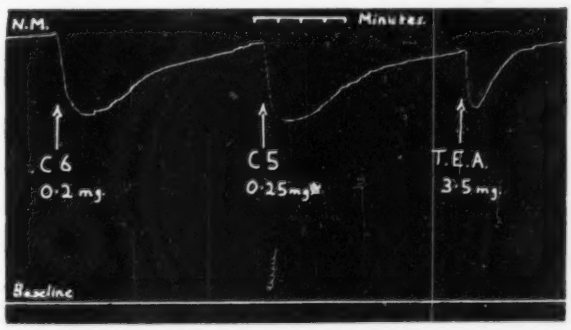

FIG. 10.—Cat, chloralose. Record of sustained contraction of nictitating membrane excited by stimulation of cervical sympathetic 10 sec. intravenous injections. Effects of 0.2 mg. C6; 0.25 mg. C5; and 3.5 mg. tetraethylammonium iodide (T.E.A.).

ammonium iodide were compared. Table VII gives a summary of those doses of these compounds and of tetraethylammonium iodide which caused roughly equal peak relaxations of the nictitating membrane. There was a considerable difference between C5 and C6 and tetraethylammonium iodide in their duration of action, the former two drugs acting more slowly and exerting their action three to four times as long as the latter, for doses which gave equal peak effects.

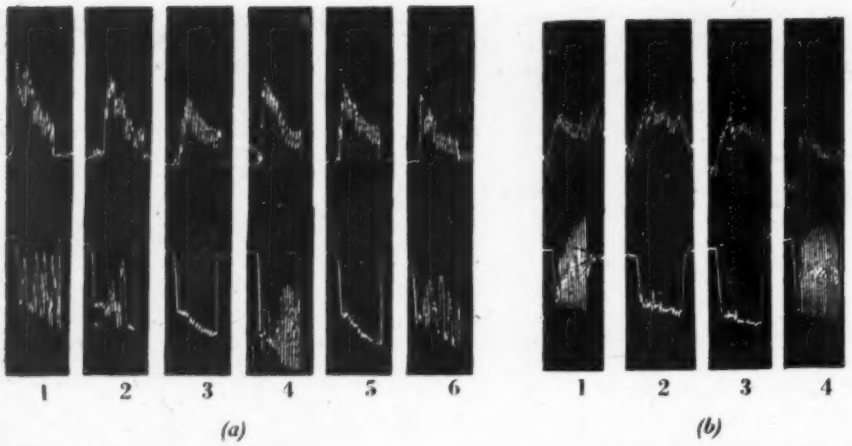

FIG. 11.—Isolated rabbit intestine. Record of length (upper tracing) and volume (lower tracing); intestine stimulated by rapid rise of intra-intestinal pressure of 3 cm. water. (*a*) 1, normal response. 2, 0.1 mg. C6 added to bath. 3, 0.2 mg. C6. 4, 15 μg., and 5, 30 μg. *d*-tubocurarine chloride. 6, normal response. (*b*) 1, Normal response. 2, 0.3 mg. C6. 3, 2.0 mg. tetraethylammonium iodide. 4, Normal response.

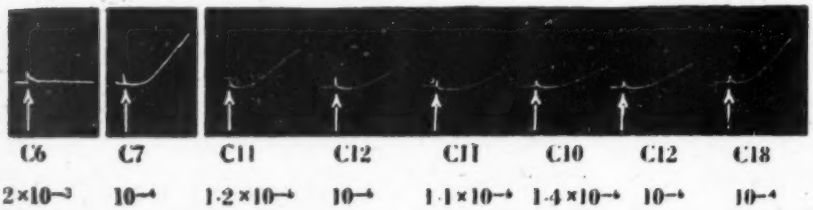

FIG. 12.—Frog's rectus. Contractions elicited by (1) 2×10^{-3} C6; (2) 10^{-4} C7; (3) 1.2×10^{-6} C11; (4) 10^{-6} C12; (5) 1.1×10^{-6} C11; (6) 1.4×10^{-6} C10; (7) 10^{-6} C12; (8) 10^{-4} C18.

To test the effect on parasympathetic autonomic ganglia, we used the technique of Trendelenburg described by Feldberg and Lin (1949). Fig. 11 shows typical tracings, and Table VII gives the relative potencies of the compounds tested. It was again observed by comparing the ease with which they could be washed out that C5 and C6 were more persistent in their action than tetraethylammonium iodine.

The intravenous injection of C6 or C5 in somewhat larger doses commonly produced a fall of blood pressure of fairly slow onset and recovery. The magnitude of the fall was closely related to the initial blood pressure level.

A search was made for stimulation of autonomic ganglia by these compounds. None of the series, however, would produce a significant elevation of the blood pressure or change of heart rate of the spinal cat even in doses twenty times those of nicotine tartrate or tetramethylammonium iodide adequate for this; nor would similar doses elicit more than a trivial contraction of the nictitating membrane. In the isolated intestine, there was a tendency for the higher members to initiate peristaltic waves (particularly C12); but this action was slight, and could be ascribed to their anticholinesterase activity.

TABLE VII

Relative potencies in causing relaxation of cat's nictitating membrane, excited by stimulation of cervical sympathetic trunk. (Arbitary scale, C6 = 100.)

Compound:	C4	C5	C6	C7	C8	Tetraethylammonium iodide
Potency:	2	80	100	10	2	5

Relative potencies on peristaltic reflex of small intestine (Arbitary scale, C6 = 100.)

Compound:	C2	C3	C4	C5	C6	C7	Tetraethylammonium iodide
Potency:	3.0	4.3	5.9	33.3	100	16.7	14

Stimulation of skeletal muscle

It was at first supposed that it would be possible to show on the frog's rectus an antagonism by C10 of the effects of acetylcholine similar to that exerted by *d*-tubocurarine chloride. It was found, however, that C10 itself stimulates the frog's rectus to contraction. Whether applied in low or high concentrations, and whether allowed to act briefly or for 10–15 min., no signs of any other than stimulant activity appeared. Indeed, so far from antagonizing acetylcholine, it usually potentiated its effects slightly, probably because of C10's weak anticholinesterase activity.

The contraction produced by C10 and its neighbours (Fig. 12) differs in several respects from that caused by acetylcholine (Fig. 14); it has a slower onset, and does not so readily reach a plateau; after C10 has been washed out, a longer time is

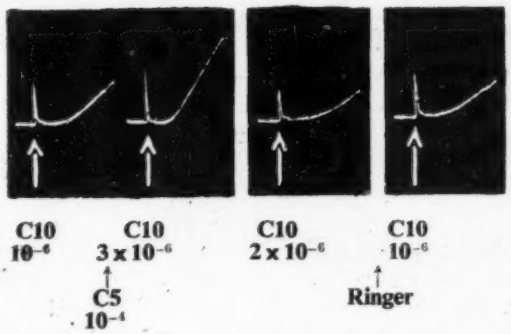

FIG. 13.—Frog's rectus. Antagonism of contracture due to C10 by C5. (1) 10^{-6} C10; (2) 3×10^{-6} C10; (3) 2×10^{-6} C10; (4) 10^{-6} C10. During (2) and (3) Ringer contained 10^{-4} C5.

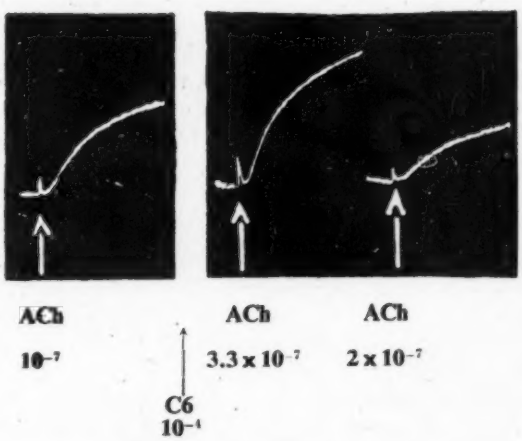

FIG. 14.—Frog's rectus. Antagonism of contracture due to acetylcholine by C6. (1) 10^{-7} ACh; (2) 3.3×10^{-7} ACh; (3) 2×10^{-7} ACh. During 2 and 3, Ringer contained 10^{-4} C6.

needed to obtain proper muscular relaxation. Its dose-response curve is, however, not dissimilar to that of acetylcholine and it resembles acetylcholine in one important respect, that its action is also antagonized by *d*-tubocurarine chloride and by fairly high concentrations of atropine (10^{-4}).

Measurements were made of the relative potency of the members of the series with respect to each other and to acetylcholine (Table VIII). During this part of the experiment, the fact mentioned above emerged, that the shorter chain compounds, which are inactive in causing contractures, were nevertheless exerting an action on the muscle, since they diminished the contractions elicited by the longer chain compounds (Fig. 13). The antagonism to C10 was a maximum with C5, closely rivalled by C6. Table IX gives the concentrations of the members tested which caused 50 per cent inhibition of C10. It was also observed that C5 and C6 can antagonize the contracture produced by acetylcholine (Fig. 14).

TABLE VIII

Relative potencies in stimulating frog's rectus abdominis. (Arbitary scale, C12 = 100.)

Compound:	C6	C7	C8	C9	C10	C11	C12	C18	Acetyl-choline
Potency:	0.1	1.3	7.1	36	71	91	100	1.3	1,500

TABLE IX

Inhibitory concentrations against C10, tested on frog's rectus. (Concentration causing 50 per cent inhibition.)

Compound:	C4	C5	C6
Concentration:	8×10^{-5}	5.0×10^{-5}	5.5×10^{-5}

The stimulation of skeletal muscle could also be demonstrated in the cat (Figs. 4 and 9). In these experiments a few μg. of C10 were injected rapidly into the artery of a tibialis prepared for close arterial injection, during an intermission of stimulation; just as after a similar injection of acetylcholine there was a fast contraction of the muscle which followed the injection so rapidly as to seem synchronous with it. After the rapid contraction is complete, there is often left a small residual contraction for a few seconds, which is followed by the onset of neuromuscular block. Roughly, the dose of C10 required to produce a twitch of the muscle is one which produces a substantial neuromuscular block; smaller doses are ineffective in both respects, and larger doses produce a larger twitch before long-lasting block ensues. It does not appear to be possible with any dose by this route to obtain paralysis without evidence of stimulation first, except by making the injection rather slowly.

After intravenous injections of C10 we have never seen the rapid twitch just described; but large doses may elicit a small contraction lasting 10–20 sec., of a few grammes tension. No detailed comparison with other members of the series has been made; but, in general, our experiments are not inconsistent with the ranking of potency in stimulating muscle, which we obtained on frog's rectus.

Anticholinesterase potency

Anticholinesterase activity was tested by two methods in order to distinguish between the activities of the compounds against the so-called "true" and "pseudo" cholinesterases. For the former, acetyl-β-methylcholine and laked rabbit cells

TABLE X

ACTIVITY IN INHIBITING CHOLINESTERASE

Substrate concentrations: for "true" enzyme 0.027 *M* acetyl-β-methylcholine; for "pseudo" enzyme 0.0055 *M* benzoylcholine

Compound	"True" concentration for 50% inhibition	"Pseudo" % inhibition at a concentration of 1.25×10^{-3}
C7	3.5×10^{-3}	
C8	1.0×10^{-3}	
C9	1.2×10^{-4}	
C10 ...	4.5×10^{-5}	8
C11 ...	1.8×10^{-5}	
C12 ...	5.6×10^{-6}	35
C18 ...	1.4×10^{-5}	97
Eserine ...	7.1×10^{-6}	
d-TC ...	1.1×10^{-3}	10

were used; for the latter, benzoylcholine and rabbit plasma as source of esterase. The compounds had little activity against "pseudo" cholinesterase, and it was not thought worth while to study their actions at concentrations higher than 1.25×10^{-3}. The values obtained are shown in Table X. Against true cholinesterase, however, the longer chain members had appreciable activity, and sufficient determinations were made to be able to estimate the concentration of drug causing 50 per cent inhibition of the enzyme; this figure for each member of the series is given in Table X, and has been plotted in Fig. 15. Comparable figures for eserine are included in Table X; it is about 650 times more active than C10 against true cholinesterase.

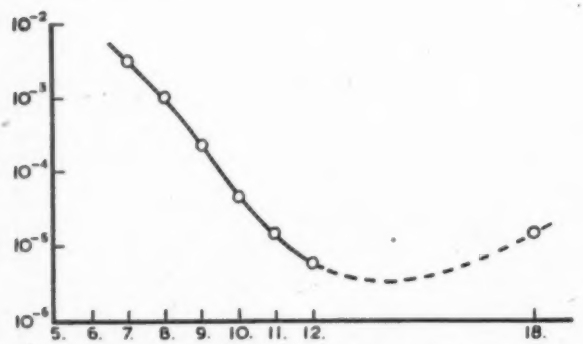

FIG. 15.—Variation of activity of bistrimethylammonium compounds against "true" cholinesterase, with varying length of polymethylene chain. Abscissa: number of carbon atoms in polymethylene chain. Ordinate: concentration for 50 per cent inhibition of the "true" cholinesterase. Substrate: 0.027 *M* acetyl-β-methylcholine.

A point of some interest is the selective paralysis of "true" cholinesterase; this, no doubt, is a function of the particular enzymes chosen. But the degree of selectivity is considerable; by extrapolation, it can be estimated that C10 is 200 times more active against "true" than against "pseudo" cholinesterases, and C12 about 600 times.

We have referred already to the potentiation of the twitch during the early stages of a block by C10. Records of the muscle action potential (Fig. 3B) show repetitive firing of the fibre in response to single shocks, similar to that well-established for eserine and di*iso*propyl fluorophosphonate. The possibility can be raised therefore that C10, although far less active than eserine *in vitro* as an anticholinesterase, becomes relatively more effective because of its lack of affinity for pseudocholinesterase in plasma. It is also possible, however, that the stimulant action of C10 on the muscle must be taken into account in explaining the fasciculations, potentiation of the twitch, and repetitive firing (cf. Zaimis, 1949).

Muscarine-like action and atropine-like action

Tetramethylammonium ion has a muscarine-like action, and it was of interest to determine whether these closely related compounds also shared it. But such activity as they possess is very slight, and could only be demonstrated easily on the isolated guinea-pig or rabbit intestine; the most active compound tested (C12) was about 1,300 times less active than acetylcholine. Atropine in a concentration of 4×10^{-9} in the bath abolished contractions due to both these drugs. No corresponding depressor action could be demonstrated when these compounds were tested on the cat's blood pressure

TABLE XI

Relative potencies in stimulating small intestine. (Arbitrary scale, C12 = 100.)

Compound:	C2	C4	C5	C6	C7	C8	C9	C10	C11	C12	C18	Acetylcholine
Potency:	<1	2.0	2.5	3.8	5	12.5	16	40	100	100	25	13×10^4

in intravenous doses up to 10 mg./kg. Table XI gives the estimated relative potencies of the series. A depressor action characteristic of certain histamine-liberators is discussed below.

Search was also made for an atropine-like action, with the response of the cat's blood pressure to acetylcholine as a test object. A dose of 10 mg. C10 did not diminish the action of acetylcholine, but rather enhanced it slightly. It appeared possible that the anticholinesterase action of C10 might be masking a feeble atropine-like action. The experiment was therefore repeated in

an eserinized animal, so that inactivation of cholinesterase should be complete before C10 was given; but it was again impossible to demonstrate any depression of the response to acetylcholine by the injection of C10. We obtained a similar result on the isolated guinea-pig's ileum. The contraction of the gut elicited by acetylcholine (2×10^{-9}; final concentration in the bath) was neither prevented nor diminished by the previous or later addition of C10 to a final concentration of 2×10^{-6}, a concentration sufficient to cause a contraction of the gut due to the C10 itself.

Release of histamine

Mention was made at the beginning of this paper that this series is not active in eliciting the delayed depressor response of the cat's blood pressure, characteristic of histamine liberation. This response can, however, be obtained, and Fig. 16

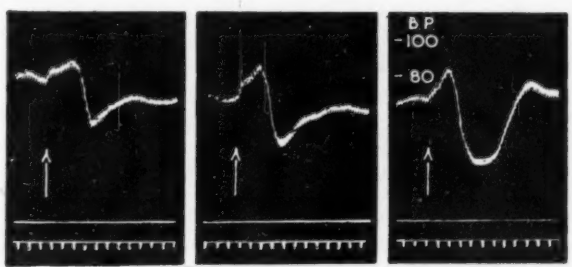

FIG. 16.—Cat, chloralose. Atropine (4 mg.), nicotine (7 mg.) previously. (1) 20 mg. C12 i.v. (2) 1 mg. propamidine isethionate i.v. (3) 5 mg. *d*-tubocurarine chloride i.v. (12 min. between successive tests.)

gives a typical illustration of the action of C12, with corresponding effects by *d*-tubocurarine chloride and propamidine for comparison (cf. MacIntosh and Paton, 1949). We have not studied this activity closely since it is so feeble compared with that in paralysing neuromuscular transmission. It appears, however, that C12 is a little more active than C10 in this respect.

Chronic toxicity

C10.—This drug was injected intravenously in a dose of 30 μg./kg. into each of three cats, and in a dose of 100 μg./kg. into each of three rabbits, six times a week for a period of five to six and a half weeks. During this time there was no consistent change in sensitivity to the drug, although day-to-day variations occurred; sometimes only weakness resulted, at other times a profound paralysis. The veins through which the injections were made (of the leg in cat, of the ear in rabbit) did not become thrombosed, and the animals maintained their normal weight and health. One rabbit and one cat, having become deeply curarized after a daily injection of C10, died before their course of six weeks was quite complete, despite artificial respiration. It must be remembered, however, that it is not easy to curarize animals repeatedly without getting occasional casualties, unless elaborate preparations for artificial respiration are made. One cat was killed as soon as it began to sicken at a time that an epidemic of agranulocytic panleucopenia was rife. Post-mortem findings showed in this animal only those changes typical of the disease. In the other animals there were no evidences of abnormality.

C6.—C6 was injected into the marginal ear vein of each of three rabbits, in a dose of 10 mg./kg., six days a week for a month. This dose is more than a hundred times the dose which is effective in man. Flushing of the ears was consistently observed, but there was no change in health or weight of the animals, and it was possible in two of the animals to make all the injections into one vein only. Post-mortem findings are given below.

If these compounds possessed any chronic toxicity it would probably be of the kind described for another aliphatic dibasic compound "synthalin" (diguanidinodecane) in which parenchymatous changes, particularly of liver and kidney, are prominent. There was no evidence either of these changes or of any other harmful action which could be ascribed to the chronic administration of these drugs.

Post-mortem findings

Sections were taken from lungs, heart, liver, gall bladder, spleen, small and large intestine, kidney, adrenals, testes, lymph glands, bone marrow, brain, and dura mater.

In the rabbits, slight peribronchiolar and perivascular lymphocytic infiltration of the lungs was observed, with occasional small areas of ulceration. These changes were also seen in control animals, and were regarded as normal.

C10. Cat 1. Chronic abscess of one mesenteric gland, but no intestinal lesion. No other abnormality.

Cat 2. (Died after daily dose.) Pulmonary oedema. No other abnormality.

Cat 3. (Sickened during epidemic of panleucopenia.) Changes in gut, lymph glands, and marrow, typical of disease. No other abnormality.

Rabbit 1. Normal.

Rabbit 2. (Died after daily dose.) Pulmonary oedema and haemorrhages. No other abnormality.

Rabbit 3. Not killed. Alive and well 1 year later.

C6. Rabbit 1. Normal.

Rabbit 2. Scanty focal patches of infiltration of meninges by lymphocytic cells. No other abnormality.

Rabbit 3. (Killed 4 months after last injection of C6.) The pia arachnoid contains an obvious excess of round cells of lymphocytic type, and similar cells accompany many of the penetrating vessels (cf. Rabbit 2). Fairly common areas of perivascular infiltration occur deeply and irregularly within the brain substance. Several foci of neuroglial proliferation are found in the cerebral hemispheres, unrelated to blood vessels, to recent destructive lesions, or to anatomical structures. Their significance is quite obscure. No other abnormality.

Antibacterial action

Although there was no reason to suppose that this series of compounds possessed an important antibacterial action, it seemed of interest to determine how far this activity varies with the length of the polymethylene chain. We are much indebted to Dr. A. T. Fuller for testing them for us. Table XII gives the concentrations of the various

TABLE XII

Inhibitory concentration of polymethylene bistrimethylammonium salts in mg./100 ml. (parts per 100,000).

Compound	Streptococci	Staphylococci	*B. coli*
C2 ...	500	>500	>500
C3 ...	500	>1,000	>1,000
C4 ...	1,000	>1,000	>1,000
C5 ...	500	>1,000	>1,000
C6 ...	500	>1,000	>1,000
C7 ...	500	>1,000	>1,000
C8 ...	750	>1,000	>1,000
C9 ...	500	1,000	>1,000
C10 ...	300, 300	1,000	>1,000
C11 ...	200	200	>500
C12 ...	150, 100	100, 200	100, 200
C13 ...	50	50	50
C18 ...	0.3	0.8	8

compounds which prevent visible growth in 18 hours at 37° C. of streptococci, staphylococci, and *B. coli.* The antibacterial activity is negligible from C3 to C12, and is only appreciable with C18. Even with the latter compound, however, the activity is only of theoretical interest.

The surface tension of aqueous solutions of the bistrimethylammonium series

The change of pharmacological action with polymethylene chain length among these compounds clearly raised the question as to how far this might be due to change in the proportion of hydrocarbon chain to polar groups in the molecule. The measurement of surface tension at an air-water interface provided a suitable method for studying this. Table XIII gives the values obtained

TABLE XIII

Depression of surface tension of water by polymethylene bistrimethylammonium salts (concentration 1 mg./c.c.).

Compound:	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C18
$\Delta\gamma\left(\frac{\text{dynes}}{\text{cm.}}\right)$:	0.4	0.4	0.1	1.3	1.0	1.4	0.6	1.8	1.3	1.3	4.0	4.3	19.3

for the depression of the surface tension of water by members of the series, in solutions containing 1 mg./c.c. of each salt. With most of the compounds there was little depression of the normal surface tension of the water. With C12 and C13, however, a distinct depression was observed, which became substantial with C18. Care was taken to control the time for which the surface was allowed to rest undisturbed before making a measurement, although the gradual fall of tension with time described for many surface-active substances was only observed with solutions of C18. The depression of tension recorded is therefore to some extent an arbitary one (measured after 10 min. had elapsed from first creating the surface), but it appeared to be within 5–10 per cent of the maximum value. It follows from these observations that one cannot look to changes in surface activity to explain variations in pharmacological actions of members below about C12.

Discussion

Species difference

One of the most remarkable properties of the members of this series, and especially of C10, was the very great variation in their activity according to the species of animal on which they were tested. Such a variation is much smaller, and in the reverse sense, with *d*-tubocurarine chloride. Variation of this sort has been described for many other onium salts, but its magnitude for C10, sufficient to make a compound of great activity in the cat appear to be of slight activity in the rat, possesses few parallels. The species variation in the sensitivity to histamine is, perhaps, the nearest to it, in that cats are relatively sensitive and mice and rats highly resistant. But this variation in sensitivity to histamine receives a possible explanation (Emmelin, 1945) in the respective plasma histamine levels of these animals: it is reasonable that those with plasmas rich in histamine should be resistant to it, since even cats can be accommodated to sustained histaminaemia. No such circulating onium salt has, however, yet been demonstrated, and we

have obtained no evidence of adaptation to the effects of C10.

Although, then, there appears to be no ready explanation of the species variation, its practical importance deserves emphasis. Any investigation of new curarizing agents must necessarily be made with more than one species. Further, even with comparative assays of one substance, it may be necessary to establish that the dose-response relations do not vary with different species ; and with assays of substances possibly containing several agents, it will be essential to be sure that all the test methods used are sensitive to all the agents involved. The properties of C10 are a strong justification for including experiments on cats at an early stage in any investigation—particularly since later trials have shown that of all the species tested the cat most closely resembles man (Organe, Paton, and Zaimis, 1949).

The antagonism of C5 and C6 to C10

The characteristics of this antagonism may be briefly summarized as follows: it is a reversible antagonism to the action of one active compound displayed by a closely related but inactive homologue. These characteristics, and the other evidence we have presented, are typical of antagonism by competitive inhibition (cf. Woolley, 1947).

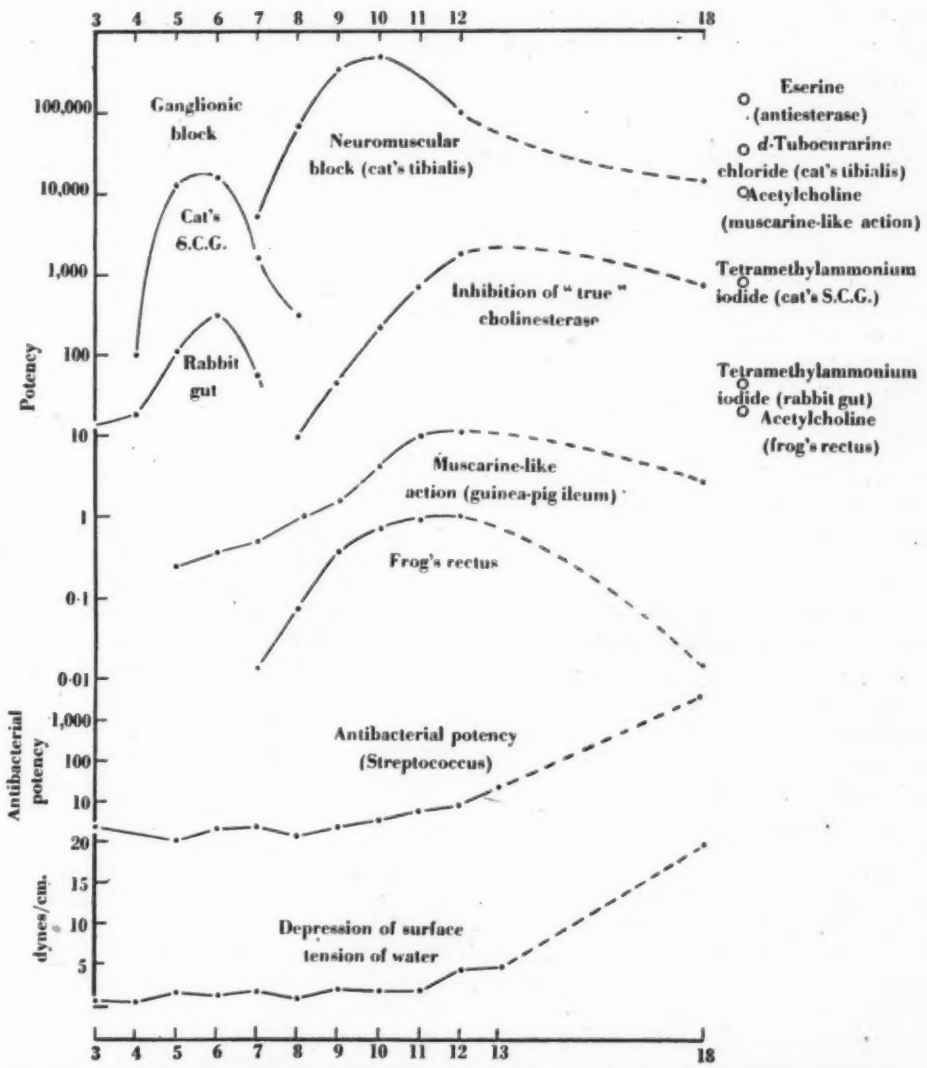

FIG. 17.—Summary of the main pharmacological actions of the bistrimethylammonium series. Abscissa: number of carbon atoms in polymethylene chain. Ordinate: logarithmic scale of potency, with arbitrary origins.

For instance, the failure of C5 to antagonize large doses of C10 is readily explained as being due to inability to achieve a sufficiently high ratio of C5 to C10 for successful competition before C5 begins to depress neuromuscular conduction itself.

Although this type of antagonism is well known it appears to be a new phenomenon in the field of neuromuscular block. We have not analysed it in any detail. But, for reasons which will be clear from what follows, we do not believe that antagonists of this kind exist for most curarizing compounds, but only for those substances which produce neuromuscular block in the same way as C10.

Relation between chemical structure and pharmacological action

We have collated the bulk of the material reported in this investigation in Fig. 17. All the main activities of the members of the series are there represented, relating their potency to their chain length. (There is no particular significance in the position of the curve for any one activity on the potency scale, the origin on the scale of ordinates being quite arbitrary; but this scale is logarithmic, so that the slopes of the curves are comparable at all points.) Together with this are plotted the measurements of the depression of the surface tension of aqueous solutions of the compounds.

These results may be roughly summarized as showing three maxima for pharmacological activities: for ganglionic action at C5–C6; for neuromuscular blocking action at C10; and a less well defined maximum for stimulation of frog's rectus, muscarine-like, and anticholinesterase activity around C12. These maxima are sufficiently distinct and striking to require separate discussion.

The easiest of these to explain is that in the region of C12. We have, here, bisquaternary compounds of increasing chain length whose activities (muscarine-like and in stimulating frog's rectus) steadily approach those of the analogous monoquaternary salts (exemplified by tetramethylammonium ion). Such a tendency is not unexpected, and occurs in other connexions. Thus it has been observed with aliphatic diamines, $NH_2(CH_2)_nNH_2$, that the shorter members ($n=6$ or 8) are substrates of diamine oxidase, and the longer members ($n=14$, 16, or 18) lose this activity but become instead substrates for mono-amine oxidase (Blaschko and Duthie, 1945). Again, MacIntosh and Paton (1949) found that the long-chain diamine with $n=16$ had a mixed pressor and depressor effect on the cat's blood pressure, characteristic both of the aliphatic monoamines and of the histamine-liberating diamines. On the other hand, as the chain length of these bisquaternary salts increases certain physical properties of the salts (which we have measured by their activity in depressing the surface tension of water) become more prominent. It is interesting that this surface activity begins to appear at C12, the point at which increase in pharmacological activity on frog's rectus and guinea-pig's intestine almost ceases. We suggest, therefore, that this maximum near C12 represents the balance-point between an increase in activity as the compounds approximate to an effectively monoquaternary state and a fall in activity as the hydrocarbon moiety of the molecule becomes large enough to influence the action of the compound.

The variations of antibacterial potency, reaching a maximum (so far as this series extended) at C18, obviously parallel directly the changes in surface activity. Similar examples, for instance among the alkyl resorcinols, are well recognized, and the relationship between surface activity and antibacterial potency is discussed by Work and Work (1948).

In quite a different category is the maximum at C10; this occurs at a point where changes in surface activity are not yet significant, although the rate of change of potency with chain-length is very rapid round the peak, especially in the C6–7–8 region: here alteration of chain length by one carbon atom causes a change in potency by a factor of about 20 times. It is of interest that approximately the same distance separates the nitrogen atoms in C10 as in *d*-tubocurarine chloride and in Bovet's compounds (3381 R.P. and 3565 R.P.) despite the differences between C10 and these substances. But it may be questioned whether this distance is specific for activity at the neuromuscular junction. Thus $n=10$–11 is optimal for histamine release by diamines (MacIntosh and Paton, 1949), and synthalin (diguanidinodecane) is the most active member of its series in causing hypoglycaemia.

The sharpness of the peak raises the question as to why the length of the polymethylene chain should be so critical, and certain comments may be made. First, C10 is at least 100 times more active in causing neuromuscular block than tetramethylammonium or trimethyldecylammonium salts (cf. Ing and Wright, 1931); it seems necessary to postulate therefore that both ends of C10 are active, presumably (from the symmetry of the molecule) in the same way. Further, it is unlikely

TABLE XIV

SYNOPSIS OF DIFFERENCES BETWEEN C10 AND *d*-TUBOCURARINE CHLORIDE

	C10	*d*-Tubocurarine chloride
1. Species sensitivity	Cat > Rabbit > Monkey > Mouse > Rat $\frac{\text{Activity in cat}}{\text{Activity in rat}} \simeq 200$	Rat > Mouse > Rabbit > Cat $\frac{\text{Activity in cat}}{\text{Activity in rat}} \simeq 0.5$
2. Muscle selectivity	Paralyses respiratory muscles less than limb muscles in cat	Paralyses respiratory muscles more than limb muscles in cat
3. Character of muscle response to tetanic stimulation during block	Well-sustained contraction	Poorly sustained contraction
4. Effect of KCl or of a tetanus on block	None	Decurarization
5. Effect of antiesterases on block	No antagonism	Decurarization
6. Effect of C5 on block	Antagonized	No antagonism
7. Effect on striated muscle ...	Elicits contracture of frog's rectus (and potentiates acetylcholine). Elicits twitch of cat's tibialis	Elicits no contraction of cat's tibialis or of frog's rectus; antagonizes acetylcholine on frog's rectus
8. Effect of *d*-tubocurarine chloride administered previously	Antagonized (both on cat's tibialis and on frog's rectus)	Potentiated. (Also potentiated by C10 previously)
9. Effect of ether anaesthesia ...	Antagonized	Potentiated
10. Effect on demarcation potential of tibialis muscle	Depressed	Not depressed

that the terminal groups ever approach each other to any great extent, since both carry strong positive charges. It is reasonable to suppose, therefore, that the molecule when active is extended on some cell-membrane. Finally, it is difficult to avoid the conclusion that the extreme sensitiveness of the activity of the series to chain-length implies some rather specific "fit" between the extended molecule and its effector site. It is tempting then to suppose that the effective distance between the terminal groups (of the order of 10Å, allowing for some freedom of movement of the chain) corresponds to the spacing of some regularly recurring receptor groups, or to the commonest inter-receptor distance in a random array of receptor groups. A recurrent acidic residue on a polypeptide chain would provide an array of the type required. Such a theory, indeed, explains the shape of the peak at C10, with its rapid cut-off at C6–C7 and slower decline from C12 onwards; for a chain less than the critical length would be entirely unable to straddle the required distance, although a longer chain would, occasionally at least, be able to adopt a configuration with its terminal groups sufficiently close.

Similar considerations apply, of course, to the peak for ganglionic activity; but too little is known about such compounds to elaborate the argument. Other considerations and anomalies raised by the activities of C5 and C6 will be referred to in another paper. It is worth mentioning here, however, that C10 and C5–C6 provide yet another and remarkable example of the dissociation of activities in neuromuscular and ganglionic block. A dose of C10 more than a hundred times that adequate for neuromuscular block fails to paralyse the superior cervical ganglion; a dose of C5 more than a hundred times that adequate for ganglionic block fails to paralyse the neuromuscular junction.

Mode of action of C10

In the following discussion we shall refer only to C10, since of the compounds studied it is the most active as a neuromuscular blocking agent; but we have no reason to doubt, and much evidence to support the presumption, that its neighbours act in the same way. The experiments we have described demonstrate that C10 possesses those properties classically regarded as "curarizing." Like *d*-tubocurarine chloride, it paralyses neuromuscular transmission only, leaving nervous

conduction unaltered, and permitting the muscle to respond to direct stimulation ; it prevents the effect of a close arterial injection of acetylcholine ; it does not interfere with the release of acetylcholine. But apart from these resemblances, there are differences which we believe to be sufficiently important to enumerate in some detail (Table XIV).

These differences are more radical than those among, say, *d*-tubocurarine chloride, its methyl ether or other related alkaloids, and the compounds studied earlier by Bovet and his colleagues. Among these there occur variations in potency, in duration of action, in activity in releasing histamine, in ganglionic activity, or in antiesterase activity. But apart from these quantitative differences (which exist also between curare and C10) there appears no evidence that the modes of action of all these compounds differ fundamentally in any way. Their actions in causing neuromuscular block, like those of curare, fit conveniently into the theory that they act by competing with the acetylcholine liberated at the nerve terminals for effector sites on the motor end-plate.

This is not true for C10. Thus agents delaying the destruction of acetylcholine do not antagonize its action. (It might be suggested that this is so because C10 itself already partially inactivates the cholinesterase around the end-plate ; but the weakness of C10 as an anticholinesterase, and the absence of any antagonistic action by C10 to that of *d*-tubocurarine chloride, make it unlikely that more than a small part of the cholinesterase concerned is inhibited by the doses of C10 used.) On the other hand, C5 is a successful antagonist, in a fashion strongly suggestive of a competitive inhibition. If C10 competes for effector sites with acetylcholine, and if C5 competes with C10 for the same sites, then C5 itself should be able to compete effectively with acetylcholine ; and it should hence possess a blocking action at the junction when given in a dose effective in antagonizing C10 ; this in fact it lacks. Only by additional assumptions about the stability of various drug-effector complexes can the properties of C5 be reconciled with a " competitive " theory of the action of C10. Another important difference lies in the ability of C10 to stimulate skeletal muscle, in concentrations little greater than those in which acetylcholine is effective. This, together with the fact that C5 is an antagonist to C10 both when stimulating muscle and when causing neuromuscular block, indicates that the role of C10 is not that of an inactive competitor with acetylcholine but that it is itself active in some respect at the neuromuscular junction.

We have described the way in which a muscle, in which partial block has been produced by C10, characteristically sustains its response to tetanization of its motor nerve. It is equally characteristic of the partially curarized muscle that it cannot maintain a tetanic response. It is out of place to discuss fully this difference here ; it may, of course, be due to some peculiar property possessed by *d*-tubocurarine chloride which has not yet been revealed ; but it also raises the possibility that the block produced by C10 is at some other part of the junctional system than that at which *d*-tubocurarine chloride produces block. The strange interaction between the two compounds suggests the same possibility. Whatever the explanation of the ability of *d*-tubocurarine chloride to antagonize C10, and of the inability of C10 to do the same to *d*-tubocurarine chloride, it is difficult to account for these facts on the supposition that the two compounds block at the same point.

The arguments we have presented suggest, therefore, that C10 produces neuromuscular block by initiating some active response in the end-plate or muscle fibre ; and that this block may arise at a part of the junctional system different from that blocked by *d*-tubocurarine chloride. An active response of the kind suggested has, indeed, been demonstrated, as a depolarization of the muscle fibre (Brown, Paton, and Vianna Dias, 1949) ; and this represents an additional distinction of C10 from curare which we have included in Table XIV. The relationship of this depolarization to the neuromuscular block remains, however, to be elucidated. We wish, at the moment, to do no more than draw attention to the striking differences between the two drugs, and to indicate the possibility of a fundamental divergence in their mode of action.

Finally, certain points may be mentioned concerning the differences in chemical structure which underlie the differences between " C10-like " and " *d*-tubocurarine-like " compounds. The information required to make a detailed comparison is not yet available. (For references, see Bovet *et al.*, 1949 ; Paton, 1949). But one may take, as a rough guide, resistance to antiesterases and stimulation of frog's rectus to be typical of C10 ; and sensitivity to antiesterases and antagonism to acetylcholine on frog's rectus abdominis to be typical of *d*-tubocurarine chloride. It then appears that, of the compounds so far adequately studied which cause neuromuscular block, only tetramethylammonium ion and its trimethyl-alkyl analogues, and succinylcholine (our experience with which agrees with that of Bovet *et al.*, 1949) and its

related aliphatic choline esters, resemble C10 ; the other compounds, including relatives of *d*-tubocurarine chloride, the erythroidines, and many of the compounds studied by Bovet and his colleagues, all resemble *d*-tubocurarine chloride. It is remarkable and suggestive that this division is also between aromatic compounds of widely variable structure on the one hand and, on the other, aliphatic compounds containing methyl-substituted quaternary nitrogen.

Summary

1. The pharmacological properties of a series of polymethylene α-ωbistrimethylammonium iodides with 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 18 carbon atoms in the polymethylene chain have been studied.

2. The decane derivative (C10) causes neuromuscular block in the cat in doses of 30 μg./kg. intravenously, and is the most active member of the series in this respect. During such block, the excitability of nerve and muscle is retained, the effects of acetylcholine injected arterially are paralysed, and acetylcholine release by motor nerve terminals is not prevented. Tetanization of the motor nerve or injection of acetylcholine or potassium neither diminishes nor deepens the block. A phase of potentiation of the muscle twitch, fasciculations of the muscle, and repetitive responses to single nerve volleys precedes the block. During a partial block, tetanization of the muscle leads to a well-sustained contraction.

3. In the cat, profound neuromuscular block of the tibialis muscle may be caused by C10, without paralysis of respiratory muscles. With *d*-tubocurarine chloride respiratory paralysis accompanies or may precede paralysis of tibialis.

4. C10 is 50–100 times less active by mouth than by intravenous injection. Its activity in cats anaesthetized with chloralose or pentothal is similar to that in the unanaesthetized cat ; but ether appears to antagonize it slightly.

5. Sensitivity to C10 varies greatly with species : in order of decreasing sensitivity, the series is cat-man - rabbit - monkey - mouse - rat. For *d*-tubocurarine chloride by the same tests, the variation in sensitivity is much smaller and decreases : rat-mouse-rabbit-cat.

6. The action of C10 is not antagonized by anticholinesterases. The pentane and hexane homologues (C5, C6) are effective antagonists ; the antagonism appears to be by competitive inhibition. Previous administration of *d*-tubocurarine chloride also reduces the effectiveness of C10.

7. C10 and its neighbours elicit a contracture of the frog's rectus abdominis muscle, and do not antagonize the contracture elicited by acetylcholine. C5 and C6 do not elicit such a contracture, but antagonize the actions both of C10 and of acetylcholine. C10 can also elicit a twitch of cat's tibialis muscle if a small dose is given by close arterial injection.

8. Members of the series, particularly C5 and C6, are able to block ganglionic transmission. Higher members possess a weak anticholinesterase action, which is more active against " true " cholinesterase than against " pseudo " cholinesterase. They also possess a weak muscarine-like action, a weak power of releasing histamine, and a feeble antibacterial action. No significant atropine-like action, or ability to stimulate autonomic ganglia, was detected. With the exception of C18, members of the series display negligible activity in depressing the surface tension of water.

9. The pharmacological properties of the series have been related to the length of the polymethylene chain. This reveals four features : (1) a sharp maximum for potency in ganglionic block at C5–C6 ; (2) a sharp maximum for potency in neuromuscular block at C10 ; (3) a less well-defined maximum for muscarine-like activity, stimulation of frog's rectus, and anticholinesterase activity about C12 ; (4) a rise in antibacterial activity and surface activity beginning about C12 and still increasing at C18.

10. The significance of the species difference, of the competitve antagonism of C5 for C10, of the variation of activity with length of polymethylene chain, and of the differences between C10 and *d*-tubocurarine chloride is discussed.

It is a pleasure to express our indebtedness to Dr. Harold King for his interest and guidance throughout this investigation, and to our colleagues for many fruitful discussions and criticisms.

We are also indebted to Dr. T. S. Work for advice on determining anticholinesterase activity, to Miss Bashford for assistance in these determinations, and to Dr. J. Niven for the histological examinations.

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THE ASSAY OF SUBSTANCES FROM THE ADRENAL MEDULLA

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Much recent work has been devoted to *nor*-adrenaline. There is evidence that this substance is present in extracts of adrenergic nerves (Euler, 1946, 1948) and liberated when the nerves to the cat's spleen are stimulated (Peart, 1949). The discovery by Holton (1949) that large amounts are present in extracts of adrenal medullary tumours directed attention to the adrenal glands themselves. The present investigation was undertaken with the object of applying the methods described by Gaddum, Peart, and Vogt (1949) to blood obtained from the adrenal veins. While it has been in progress evidence has been obtained by others that *nor*adrenaline is present in extracts of the adrenal glands (Holtz and Schumann, 1948; Euler and Hamberg, 1949; Goldenberg, Faber, Alston, and Chargaff, 1949; Tullar, 1949), and liberated from these glands when the splanchic nerves are stimulated (Bülbring and Burn, 1949). The results recorded below are in agreement with these conclusions.

METHODS

Collection of blood.—Cats were anaesthetized with chloralose (40 mg. per kg.) after preliminary ether. Blood pressure was recorded with a mercury manometer. Most of the cats were eviscerated. The nerves in the mesentery were stimulated after the mesenteric arteries had been tied, but while the portal vein was still open; this transferred blood into the rest of the cat, so that the blood pressure usually remained high in spite of the removal of samples for testing. The kidneys were removed or the renal vessels tied. The adrenolumbar veins were tied just peripherally to the adrenal glands. All other branches of the inferior vena cava below the liver were tied. Both the aorta and the inferior vena cava were tied below the renal vessels, and a cannula inserted in the vena cava just above the ligature. When the vena cava was closed by a clip near this cannula adrenal blood flowed to the heart. When this clip was moved to a point above the origin of the adrenolumbar veins, blood flowed out through the cannula and was collected.

Various precautions were taken to prevent clotting and the release of interfering substances. The cannulae were made unwettable with Teddol, obtained from the British Thomson-Houston Co. This was diluted to 5 per cent with CCl_4, and the cannulae were rinsed in this fluid and then washed with water. Centrifuge tubes were coated with paraffin wax and cooled in ice-water. Heparin (3,000 units per kg.) was injected into the cat, and 10 units per ml. of blood were placed in each centrifuge tube. Blood was run into these tubes and rapidly centrifuged. Pharmacological tests were applied to the plasma, which was kept in the refrigerator until tested.

Stimulation.—The splanchnic nerves were divided early in the experiment. They were stimulated with an alternating current (50 cycles, 5–10 volts).

Preparation of extracts.—At the end of some experiments extracts of glands were prepared. After careful dissection the glands were weighed, immersed in 10 ml. 0.15*N* HCl, ground with sand, and kept in the refrigerator. Shortly before the assay an aliquot of the supernatant fluid was diluted with saline and the *p*H was adjusted to 5 with solid $NaHCO_3$. After standing, this fluid was filtered and the filtrate used for the test, further dilutions being made if necessary with saline containing ascorbic acid (10^{-4}).

Assay.—Samples of plasma extracts were compared with solutions of synthetic *l*-adrenaline (B.D.H.) and *dl-nor*adrenaline hydrochloride (Sterling Winthrop). The results for *nor*adrenaline are expressed in terms of *l-nor*adrenaline, the activity of the *d-nor*adrenaline being neglected.

The method used was based on that of Jalon, Bayo, and Jalon (1945). Rat's uterus, or colon, is suspended in a solution of the following composition (g./l.): NaCl 9. KCl 0.42. $CaCl_2$ 0.06, $NaHCO_3$ 0.2, glucose 0.5 at 30° C. Contractions are produced every 2 min. by a choline ester, and the assay depends on the inhibition of these contractions by adrenaline or *nor*-adrenaline.

The addition of the choline ester was made automatically by means of the apparatus shown in Fig. 1; it is based on that of Schild (1946, 1947). The uterus or colon is suspended in a small bath (2 ml.). Contractions are produced at intervals of 125 sec. by replacing the solution in the bath by a similar solution

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containing carbachol. This substance is more suitable than acetylcholine for this purpose since it is stable even at *p*H 8 for days, whereas acetylcholine is hydrolysed rapidly in the reservoir. The carbachol solution is allowed to act for 30 sec. and is then replaced by the original solution. The concentration of carbachol is 0.5–1.0 μg. per ml. for the uterus and 0.05–0.1 μg. per ml. for the colon.

The changes are made in the following way. A cog wheel, which is rotated by clockwork, makes and breaks a contact every 5 sec. At each break a telephone uniselector moves on one place. At suitable intervals this makes contacts which activate telephone relays and these release the compression of rubber tubing and allow solutions to run in at the bottom of the organ bath. A capillary tube permanently connected with a filter pump sucks off excess fluid near the top of the bath, which is wider than the lower part. Each time that the contents of the bath are changed the fluid flows for 5 sec. The height of the reservoir (about 30–40 cm.) is adjusted so that the amount of fluid which flows in 5 sec. is about 5 times the volume of the bath.

When left to itself this apparatus produces a very uniform series of contractions. Solutions of adrenaline or other substances are added to the bath in a volume of 0.02–0.2 ml. when a signal lights up 1 min. before the inflow of carbachol. The adrenaline thus acts on the muscle for 1 min. and is not present in the bath when the carbachol is acting; this causes no serious loss in the sensitivity of the test. In the method used previously the addition of plasma to the bath was apt to cause a variable loss of fluid in froth, so that the addition of a constant amount of choline-ester produced a variable concentration in the bath. With the method described here the concentration is constant, and the froth is washed away so that it does not interfere with the contraction.

It has been found convenient to conduct tests on the uterus and colon simultaneously with the arrangement shown in Fig. 1.

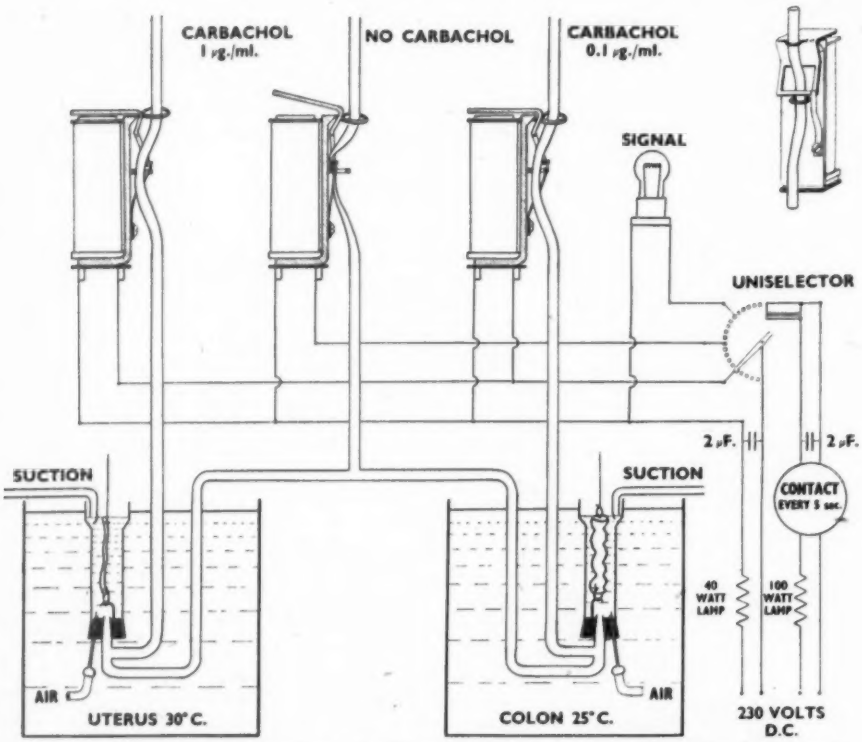

FIG. 1.—Automatic apparatus. Replaces fluid by carbachol solution for 30 sec. every 2 min.

Uteri from old rats often react slowly and it is sometimes desirable to alter the setting of the apparatus so that the carbachol is in the bath for more than 30 sec. Uteri from young rats (4–5 weeks) are generally preferable but may be too thin and weak. Satisfactory results have been obtained with such uteri by using both horns tied together, side by side.

RESULTS

In a number of experiments blood from the adrenal veins and extracts of glands were tested by the comparatively simple methods used by Gaddum, Peart, and Vogt (1949). It was easy to show in this way that stimulation of the splanchnic nerves, or the injection of acetylcholine, histamine, or KCl into the arteries, caused the release of large amounts of active substances into the plasma. When the plasma was compared with adrenaline by means of the uterus and colon the results were usually discrepant, and the discrepancy could usually be accounted for on the theory that *nor*adrenaline was present as well as adrenaline. Similar evidence was obtained with extracts, but none of this evidence was really convincing. When the percentage methylation of the mixture is small (i.e., when there is a large excess of *nor*adrenaline) the method works well, as it did in the experiments of Peart (1949). When methylation is more than 50 per cent, the method becomes inaccurate. These earlier results were considered

unsatisfactory and will not be presented in detail.

It became clear that better methods were needed, and various methods were tried. King (1949) has described a test for adrenaline in which the rat's uterus is bathed in a solution containing more than the usual amount of potassium. We have confirmed the fact that this provides a sensitive test which has the advantage of simplicity. On the other hand, it is slower than de Jalon's method, and did not appear to be more sensitive. In a small bath, solutions with a low potassium content inhibit the uterus even when they contain no adrenaline. It is not easy to avoid effects due to the potassium content of the solutions tested, and the use of this method was therefore abandoned.

The purpose of this paper is to describe experiments by de Jalon's method using the semi-automatic bath and statistics. The result depended on the measurement of the effects of two doses of the standard preparation (S_1 and S_2) and two doses of the unknown preparation (U_1 and U_2) and the use of the formulae proposed by Gaddum (1933). The application of this technique to isolated organs was first discussed by Schild (1942); our calculations of the errors were made by the simplified method described by Bliss (1944) and Noel (1945). The results are calculated from differences between effects obtained at about the same time and are thus independent of slow changes in the size of the response.

The validity of the calculations depends on the assumption that the effect is linearly related to the logarithm of the dose. Various methods of measuring the effect were tried. The response to adrenaline depends on the difference between the effect produced under its influence and the effect which would have been produced without it. An estimate of the latter quantity can be obtained by measuring on the drum the last contraction, before the addition of the adrenaline (or other substances) in each of a set of four doses. The average of these four figures is taken as the effect of carbachol alone during this time. The heights of the contractions in the presence of S and U are calculated as a percentage of this quantity. This method of measurement makes allowance for the fact that the response of the uterus to carbachol tends to diminish in the course of an hour or two. Fig. 2 shows the results of an experiment calculated in this way. It also shows the results of a similar experiment with the colon. Since the base-line is unstable with this organ the measurements were made from an artificial base-line fixed at an arbitrary level through the bottom of the tracing.

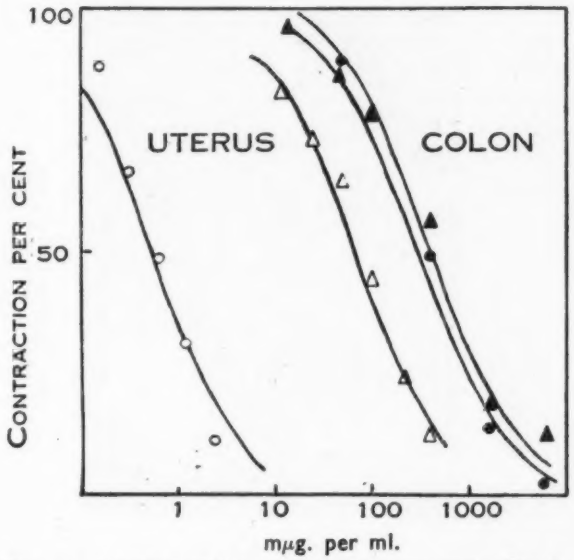

FIG. 2.—Vertical scale: contraction per cent, due to carbachol. Horizontal scale: concentration of drugs in bath. Circles: adrenaline. Triangles: *nor*adrenaline; white: rat's uterus; black: rat's colon. Theoretical curves (see text).

Clark (1937) has drawn attention to the fact that the relation between the dose of acetylcholine or adrenaline and some of their effects on plain muscle can be explained in terms of the mass laws. In the simplest case the curve connecting these quantities is a hyperbola, and the S-shaped curve connecting the effect with log dose has a characteristic slope. The curves drawn in Fig. 2 have been calculated on this theory. It will be seen that the observations fit the curves fairly well except for those showing the effect of adrenaline on the uterus, which would be better fitted by a steeper curve. The calculations might perhaps be based on the assumption that the points lie on

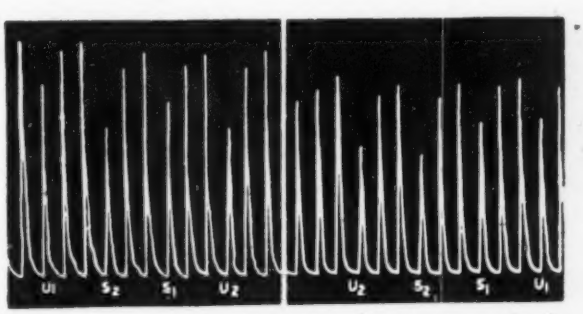

FIG. 3.—Rat's uterus. Exp. 50. Carbachol every 2 min. Effects of standard adrenaline (1 and 2 mμg.) and unknown solution. Ratio of doses $S_2/S_1 = U_2/U_1 = 2$.

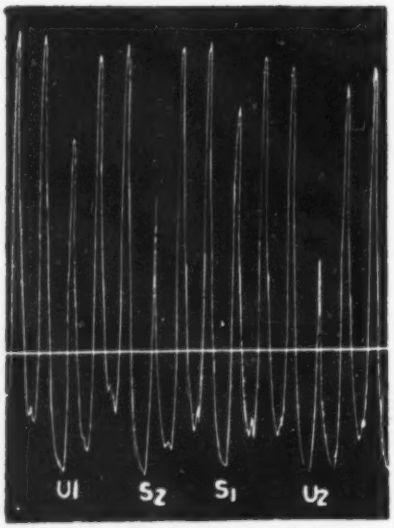

FIG. 4.—Rat's colon. As Fig. 3, but doses of adrenaline 400 and 800 mμg.

these curves, but in the present state of knowledge it seems safer to adopt the simpler approximation that the curves are straight lines ; this seems to be nearly enough true for effects between 20 and 80 per cent. It is fortunate that the curves for adrenaline and *nor*adrenaline have about the same slope. If this had not been so, the results would have been very difficult to interpret.

TABLE I

EFFECTS ON UTERUS (EXP. 50)

Sets	Doses	Contraction before U or S		Contraction after U or S	
		mm.	Mean	mm.	Per cent of mean
1	U_1	74		60	84
	S_2	73		46	64
	S_1	70		54	76
	U_2	69	71.5	46	64
2	U_2	70		46	70
	U_1	65		54	82
	S_2	66		44	67
	S_1	63	66	50	76
3	U_2	62		41	67
	U_1	61		50	82
	S_1	61		46	75
	S_2	61	61	37	61
4	U_2	62		39	65
	S_2	59		37	62
	S_1	59		47	78
	U_1	60	60	48	80

Less elaborate methods of measuring the effect can be used in routine tests. The results with the uterus discussed below were actually calculated as percentages, but direct calculations from the heights of the records in mm. would probably be equally satisfactory, since the method of calculation used is designed to eliminate errors due to a slow change in the response of the tissue. In the experiments with the colon the calculations actually were made directly from measurements of the height of the response above an artificial base-line. For this purpose, it is unnecessary that this base-line should be at the bottom of the record and it was placed at any convenient height (cf. Fig. 4). The response of the colon to carbachol was well maintained for six to eight hours, though there was a tendency for the muscle to lengthen gradually. The effect of this change is eliminated by the method of calculation.

TABLE II

CALCULATIONS FOR ASSAY ON UTERUS (EXP. 50)

N =	4 Sets of results	1	2	3	4
S_1	Effects of standard	76	76	75	78
S_2		64	67	61	62
U_1	Effects of unknown	84	82	82	80
U_2		64	70	67	65
D_1	U_2-S_2	0	3	6	3
D_2	U_1-S_1	8	6	7	2
D_3	U_2-U_1	−20	−12	−15	−15
D_4	S_2-S_1	−12	−9	−14	−16
y_1	D_1+D_2	8	9	13	5
y_2	D_3+D_4	−32	−21	−29	−31
y_3	D_1-D_2	−8	−3	−1	1
T_1	$S(y_1)$	Sample difference			35
T_2	$S(y_2)$	Dose difference			−113
T_3	$S(y_3)$	Slope difference			−11
I	Log ratio of doses	Log 2			0.3010
M	T_1I/T_2	Log potency of U/S			−0.09323
R	100 antilog M ...	Potency of U (% of S)			80.68
b	$T_2/2IN$	Slope			−46.927
A	$S(y_1)^2+S(y_2)^2+S(y_3)^2$	—			3,681
B	$S(T_1^2+T_2^2+T_3^2)$	—			14,115
s	$\sqrt{(A-B/N)/12(N-1)}$	S.D. of effect			2.0566
λ	s/b	S.D. of log dose			0.04383
λ_M	$\lambda\sqrt{(1+T_1^2/T_2^2)/N}$ (1-antilog $\lambda_M)R$ or 2.303 $\lambda_M R$	S.D. of M S.D. of R			0.02294 4.3
t	$T_3/2s\sqrt{N}$	—			1.34

TABLE III

ESTIMATES OF ERROR

Exp.	Uterus			Colon		
	λ	λ_M	Coefficient of variation per cent	λ	λ_M	Coefficient of variation per cent
40x	0.0778	0.0403	9.7	0.145	0.0744	18.7
40y	0.0425	0.0217	5.2	0.112	0.0572	14.1
48	0.0615	0.0540	11.3	0.179	0.0950	24.5
49	0.0597	0.0314	7.5	0.101	0.0505	12.3
51	0.0519	0.0251	5.9	0.093	0.0470	11.4
50	0.0438	0.0229	5.4	0.219	0.1125	29.6
52	0.0447	0.0270	6.4	0.0717	0.0423	10.3
53(2)	0.0653	0.0382	9.1	0.0529	0.0265	6.3
53(3)	0.1097	0.0567	14.0	0.164	0.0864	22.0
Mean	0.06188	0.03527	8.3	0.1264	0.0058	16.6

The appropriate doses are chosen as the result of preliminary tests. In experiments with the uterus the ratio of the larger doses (S_2 and U_2) to the smaller doses (S_1 and U_1) is 2; with the colon this ratio is 2 or 4. These doses are given in random order; this whole set of results is repeated four times using different random orders of doses. The paper is then varnished, and the contractions measured and the calculations made. Fig. 3 shows the first and last sets of results in experiment 50 with the uterus. Fig. 4 shows one of the four sets of results in experiment 50 with the colon.

Table I shows the measurements made from the tracing shown in Fig. 3 and the calculation of the percentage effect, which is shown in the last column.

Table II shows the calculation of the result of an assay and its error. In this assay the potency of *U* was estimated as 80.68±4.3 per cent of that of *S*. The quantity *t* is used to calculate whether the slopes of the two curves are significantly different. A standard table of *t* shows that the value obtained here corresponds to a probability between 0.2 and 0.3, when there are 3(*N*–1) or 9 degrees of freedom. There is thus no evidence that the lines are not parallel.

Table III shows estimates of the error in 9 assays on uterus and colon. The quantity λ gives the best measure of the general accuracy of the test, being independent of the number of sets of results. The mean values were about 0.062 for the uterus and 0.126 for the colon. These figures may be compared with 0.043 for the assay of histamine on guinea-pig's ileum (Schild, 1942) and 0.033 for the assay of adrenaline on dog's blood pressure (Noel, 1945). The standard error of the actual assays was about 8.3 per cent for the uterus and twice as large for the colon.

The test for parallelism of the dose-effect curves was made in all 18 assays. *P* was greater than 0.05 in every assay except one, and in that one it was only slightly less. There was thus no evidence of lack of parallelism.

The calculation of concentrations in mixtures

If a solution containing a mixture of adrenaline and *nor*adrenaline is assayed against pure solutions of the two drugs using both uterus and colon, the four results may be used to provide estimates of the concentrations in the mixture, if it is assumed that the effects of the two drugs are simply additive and that no other active substances are present. The logical basis of the formulae used for this purpose is given below. Similar formulae have been devised independently by Euler (1950) and Bülbring (1949).

Let *Au*, *Ac*, *Nu*, and *Nc* denote the adrenaline-equivalents and the *nor*adrenaline-equivalents, on the uterus and colon respectively, as determined by assay, and let *A* and *N* denote the actual concentrations of the two drugs.

Let *Ru* and *Rc* denote the ratios of doses of *nor*adrenaline to the equivalent doses of adrenaline, so that $Ru = Nu/Au$ and $Rc = Nc/Ac$.

The adrenaline equivalent of the mixture should be equal to the sum of the adrenaline equivalents

of the two drugs present. We thus have the two equations

$$Au = A + \frac{N}{Ru} \qquad Ac = A + \frac{N}{Rc}$$

Whence $$N = RcRu\,\frac{(Ac - Au)}{(Ru - Rc)}$$

and $$A = \frac{AuRu - AcRc}{Ru - Rc}$$

Actually Rc is negligibly small compared with Ru, therefore

$$N = (Ac - Au)\ Rc \qquad (1)$$

and $$A = Au - Ac\frac{Rc}{Ru} \qquad (2)$$

When the percentage methylation is high it is best to use adrenaline as the main standard preparation, and the equations are given above in a form which is convenient when this is done.

The evidence that *nor*adrenaline is present in the mixture depends on the difference between the two estimates of the adrenaline equivalent (Ac and Au) and is only significant when these estimates are significantly different from one another. When this is so, formula (1) gives a convenient estimate of the concentration of *nor*adrenaline. The result with the uterus (Au) can sometimes be taken as a direct estimate of the concentration of adrenaline; equation (2) provides a correction which is negligible when the percentage methylation is high, but becomes important when this percentage falls below 20.

When the percentage methylation falls below 10, it is theoretically better to use *nor*adrenaline as the standard preparation and the following equations will then be more convenient. They are really the same as those given above.

$$A = \frac{(Nu - Nc)}{Ru} \qquad (3)$$

$$N = Nc - Nu\frac{Rc}{Ru} \qquad (4)$$

The discussion on equations (1) and (2) also applies *mutatis mutandis* to equations (3) and (4).

Table IV shows the results by this technique with known mixtures of adrenaline and *nor*adrenaline. All these assays were made by the statistical procedure outlined above, and their errors have already been discussed. It will be seen that the calculations give estimates of the concentrations of adrenaline and *nor*adrenaline in the mixture which are likely to be accurate enough for some purposes.

Table V shows the results of the application of this method to extracts of adrenal glands and to blood obtained from the adrenal veins during

TABLE IV

ASSAYS OF KNOWN MIXTURES

No. of experiment:	40x	40y	48	49	51
Adrenaline equivalent:					
Uterus	240	432	1,438	2,234	2,030
Colon	1,177	1,160	6,508	4,860	3,796
Ratio (*Nor.*/Adr.)					
Uterus (*Ru*) ...	80	80	500	37	180
Colon (*Rc*) ...	1	1	0.5	0.73	1
Adrenaline concentration:					
Calculated ...	225	417	1,431	2,138	2,009
Actual	200	500	2,000	2,000	2,000
Error per cent ...	+12.5	−16.6	−28	+11	+0.4
*Nor*adrenaline concentration:					
Calculated ...	937	728	2,530	1,917	1,766
Actual	800	500	2,000	2,000	2,000
Error per cent ...	+17.1	+45.6	+26.5	−4.1	−11.7
Total amine:					
Calculated ...	1,162	1,145	3,961	4,055	3,775
Actual	1,000	1,000	4,000	4,000	4,000
Error per cent ...	+16.2	+14.5	−1	+1.4	−5.4
Methylation per cent					
Calculated ...	19	36	36	53	53
Actual	20	50	50	50	50

stimulation of the splanchnic nerves. In all these assays the estimated percentage of methylation was high, and it was necessary to test whether the results could be accounted for on the theory that adrenaline was the only active substance present. The evidence against this theory lies in the fact that $Ac > Au$. The fourth line gives estimates of the probability that the observed differences would occur by chance owing to the error of the test. The method by which these figures were calculated may be illustrated by an example. In experiment 50 the difference between the logarithms of the two estimates = log 938/556 = 0.2201. The standard errors of the logarithms of the two estimates are 0.0229 and 0.1125 (Table III). The standard error of 0.2201 is thus $\sqrt{(0.0229)^2 + (0.1125)^2} = 0.1148$. Therefore $t = 0.2201/0.1148 = 1.92$, and P lies between 0.05 and 0.1 (9 degrees of freedom). This experiment thus provides no significant evidence of the presence of any active substance in the solution besides adrenaline. The error of the test on the colon was unusually large in this assay. On the other hand, experiment 52 provides significant evidence of the presence of some other substance in the extract of

TABLE V

ESTIMATES OF CONCENTRATIONS IN ADRENAL GLANDS AND ADRENAL VENOUS PLASMA

	Adrenal glands μg. per g.		Plasma μg. per ml.	
No. of experiment:	50	52	53(2)	53(3)
Adrenaline equivalent:				
Uterus	556	618	2.62	1.66
Colon	938	877	4.78	2.32
Probability of difference ...	0.05–0.1	ca 0.01	<0.001	0.1–0.2
Ratio (*Nor.*/Adr.):				
Uterus (*Ru*) ...	180	150	75	75
Colon (*Rc*) ...	0.21	0.9	0.33	0.43
Calculated concentration:				
Adrenaline ...	555	613	2.61	1.65
*Nor*adrenaline ...	80	236	0.71	0.28
Methylation per cent	87	72	78.5	85.5

the gland. It would clearly not be possible to identify this substance in a mixture by tests of this kind, but, if it is justifiable to assume that it is *nor*adrenaline, then the results give an estimate of the amount present.

In experiment 53 a control sample of plasma from the adrenal veins was roughly equivalent on the uterus to 0.005 μg. of adrenaline per ml. On stimulation of the splanchnic nerves, the figure rose immediately to 2.62 μg. per ml. (sample 2). Stimulation was continued without intermission for 64 min. and sample 3 was collected during the last 16 min. of this time. The results of assays on these two samples are shown in Table V. In sample 2 there was significant evidence that adrenaline was not the only active substance present. The results with sample 3 were similar, but the evidence was not significant; such results might occur with adrenaline alone. The percentage methylation appeared to rise slightly. There was no evidence in this experiment of a loss of the adrenals' powers of methylation even when the nerves were continuously stimulated for over 45 min.

Discussion

Straightforward pharmacological methods can be used to identify adrenaline or *nor*adrenaline when either is present alone or in overwhelming excess. When the percentage methylation of a mixture of these substances is 10 or more, *Au* gives a direct estimate of *A*; when it is 10 or less, *Nc* gives a direct estimate of *N*. When the percentage is about 10 both these quantities can thus be estimated directly with fair accuracy. In other cases, more complicated methods must be used. If there is doubt about the nature of the active substances present in the mixture the methods described here are not well adapted for their identification. If evidence from other sources justifies the assumption that these substances are adrenaline and *nor*adrenaline the formulae give some indication of the percentage methylation.

The presence of *nor*adrenaline in extracts of adrenals is already proved, and the percentage methylation has been estimated by chemical methods. The pharmacological methods have the advantage of sensitivity and may be useful in problems where the concentrations are low. The fact that the methods described here have given similar results to the chemical methods when applied to extracts is evidence in favour of their value when applied to lower concentrations.

The results of experiment 53 show that adrenaline was not the only substance liberated by the nerves. Acetylcholine is known to be liberated, but would be hydrolysed in the conditions of these experiments and would not produce the observed effects. It would either cause a contraction of the colon within a few seconds of being added to the bath or have no effect at all. Histamine is unlikely to be the cause of the discrepancy, since it is comparatively inactive in these tests. The release of *nor*adrenaline is the most likely explanation of this result, which thus confirms the conclusions of Bülbring and Burn (1949). It is, however, clearly desirable to separate the two substances before tests are made; a method of doing this is in course of development.

Summary

1. The concentrations of both adrenaline and *nor*adrenaline in a mixture of these drugs can be roughly determined by parallel quantitative assays on the rat's uterus and colon.

2. The results may be misleading unless they are analysed statistically.

3. Extracts of cats' adrenals and plasma collected from the cat's adrenal veins during stimulation of the splanchnic nerves both contain some other substance besides adrenaline. The results confirm the view that this is *nor*adrenaline and give an estimate of the amount.

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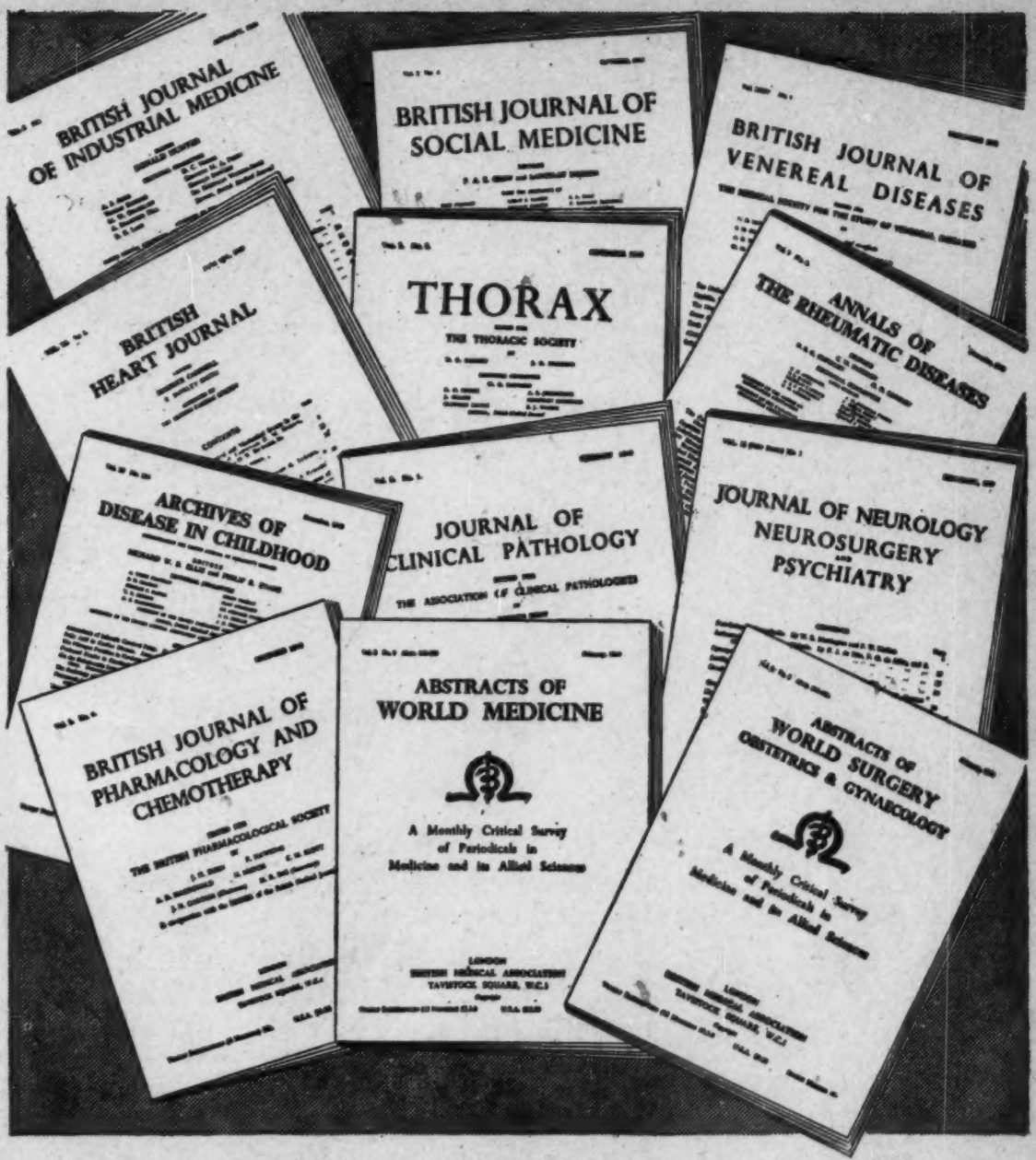
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